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THE UNIVERSITY OF ALBERTA

A CYTOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE POTENTIAL B GENOME DONORS TO COMMON WHEAT, *Triticum aestivum*.

by

© KENT BLAIR KERBY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL 1986

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Cytological and Biochemical Characterization of the Potential B Genome Donors to Common Wheat, *Triticum aestivum* submitted by Kent Blair Kerby in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

J. Kuspira
Supervisor

George Fedak
External Examiner

Date Aug 15, 1986

For Jim and Lila Kerby

and my wife Tracy

Abstract

In the past, evidence has been presented which has implicated *Aegilops speltoides* Tausch, *Aegilops longissima* Schweinf and Muschl., *Aegilops bicornis* (Forsk.) Jaub. and Sp., *Aegilops searsii* Feld. and Kis., *Triticum urartu* Thum., and *Aegilops sharonensis* Eig. as the B genome donor to *Triticum turgidum* (L.) Thell. and *Triticum aestivum* (L.) Thell.

In an attempt to identify the source of the B genome to tetraploid and hexaploid wheat, two approaches were followed. The first approach involved the isolation and determination of the amino acid sequence of the seed protein purothionin. The B genome donor should contain a purothionin with an amino acid sequence identical to the α_1 purothionin present in the B genome of the tetraploid and hexaploid wheats. None of the species examined had an amino acid sequence identical to that of α_1 purothionin. However, *A. searsii* and *A.bicornis* contain a protein which differs from α_1 purothionin by different single amino acid substitutions.

The second approach involved comparing the somatic chromosome complements from root tip cells of the putative B genome donors with the B genome chromosomes in *T. turgidum*. Since the karyotype from *T. monococcum* consists of 1 sub-terminal pair, 4 sub-median pairs, and 2 median pairs of chromosomes, and the karyotype of *T. turgidum* consists of 2 satellited pairs, 2 sub-terminal pairs, 7 sub-median pairs, and 3 median pairs of chromosomes, the B genome donor should contain 2 satellited pairs, 1 sub-terminal pair, 3 sub-median pairs, and 1 median pair of chromosomes. *A. searsii* was the only species examined that has this karyotype description.

Therefore, on the basis of the amino acid sequence of purothionin and karyotype analysis, *A. searsii* is the most likely candidate as the B genome donor to *T. turgidum* and *T. aestivum*.

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It is a pleasure to thank Dr. John Kuspira for his guidance and patience throughout the course of this investigation.

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INTRODUCTION

The *Gramineae*, or grass family, is composed of about 600 genera and over 5,000 species (Peterson 1965). The cereals are members of this family which produce large, edible grains (Peterson 1965) and provide most of mans energy and protein needs (Johnson 1984). Common bread wheat, *Triticum aestivum* L., is one of the most important cereals grown to provide these energy needs (Inglett 1983).

T. aestivum is an allohexaploid (Kihara 1924, referenced by Kihara 1982), with $2n = 6x = 42$ chromosomes (Sakamura 1918, referenced by Watkins 1930) and the genomic formula AABBDD (Kihara l.c.). Based on archeological evidence, *T. aestivum* has been cultivated for over 8,000 years (Helbaek 1949).

Genetic variation can be found in many cereal species that grow wild in nature. *T. aestivum*, however, continues to exist only under cultivation. Various workers have tried to identify the progenitor species of *T. aestivum*, hoping to draw upon the natural variation found in these wild species.

It is widely accepted that *Triticum monococcum* L. is the A genome donor to tetraploid and hexaploid wheats (Kihara, l.c.). It is universally accepted that *Aegilops squarrosa* L. is the D genome donor to *T. aestivum* (Kihara l.c. , Pathak 1940, Kihara 1944, McFadden and Sears 1946). *Triticum turgidum* (L.) Thell is an allotetraploid (Kihara l.c.) with $2n = 4x = 28$ chromosomes (Sakamura l.c.) and the genome formula AABB (Kihara l.c.).

For over 60 years, attempts have been made to identify the donor of the B genome to the tetraploid and hexaploid wheats. On the basis of morphological, ecological, cytological, protein, and deoxyribonucleic acid (DNA) analyses (referenced in the literature review), the following species have been implicated as potential B genome donors: *Aegilops bicornis* (Forsk.) Jaub and Sp., *Aegilops longissima* Schweinf. and Muschl., *Aegilops searsii* Feld. and Kis., *Aegilops sharonensis* Eig, *Aegilops speltoides* Tausch, and *Triticum urartu* Thum.

In an attempt to resolve this long standing problem, two approaches were undertaken in this study. The first approach was a biochemical study of the endosperm protein, purothionin, which has a genome specific amino acid sequence. The amino acid sequence of the purothionins found in the putative B genome donors were compared to the amino acid sequences of these proteins found in *T. monococcum*, *T. turgidum*, and *T. aestivum*.

The second approach was a cytological one. It involved karyotyping *T. monococcum*, *T. turgidum*, and each of the putative B genome donors and a comparison of these karyotypes.

Using these two approaches, it was hoped that the information obtained would help resolve this long standing problem.

Literature Review

Taxonomy of Triticum and Aegilops

On the basis of plant morphology, Schulz (1913, referenced by Percival, 1921) placed each species of Triticum into one of three groups which he called Einkorn, Emmer, and Dinkel. Vavilov (1914) found that the species placed in the above groups differed in their reaction to the rust (Puccinia triticina) and mildew (Erysiphe graminis) pathogens. The Einkorns were immune to rust and mildew, the Emmers are resistant, and the Dinkels were susceptible to both pathogens. Further support for the taxonomic proposal of Schultz came from Tschermark (1914, referenced by Percival 1921). He found that crossing two species from the same group was easier and the hybrids more fertile than crossing two species from different groups. Sakamura (1918, referenced by Watkins 1930) found that species placed in the Einkorn group contained a somatic chromosome number of 14, the species placed in the Emmer group had 28 chromosomes, and the species placed in the Dinkel group had forty-two chromosomes. This was the first report of the correct chromosome number in the diploid, tetraploid, and hexaploid wheats. During that year, Sax (1918) reported the somatic chromosome number in T. turgidum var. durum to be 28, which confirmed Sakamura's observations.

Disagreement regarding the superspecific, specific, and subspecific taxa in Triticum and Aegilops has existed since the 1920's.

The tribe Triticeae Dumort. was often called Hordeae Benth until 1954 when Pilger pointed out that the term Triticeae was used in the publication by Dumortier in 1823 while Hordeae did not appear in the literature until 1881 (Mac Key 1966). The correct section names in the genus Triticum are Monococca, Dicoccoidea, and Speltoidea Flaskb. (published by Flaskberger in 1928, referenced by Mac Key 1966) and not Diploidea, Tetraploidea, and Hexaploidea Flaskb. (published by Flaskberger in 1938, referenced by Mac Key 1966) as they were sometimes called.

Due to the economic importance of the Triticum species, conspicuous morphological differences, and not the ability to form fertile hybrids, were the primary considerations used in species classification. Often, the morphological characteristics were based on a few type specimens kept in botanical gardens. Percival (1921) recognized seven tetraploid species in the genus Triticum while McFadden and Sears (1946) listed ten species. Mac Key (1966) recognized only two tetraploid species, Triticum timopheevi (with two sub species), and T. turgidum (six sub species and four con varieties).

Eig (1929) arranged the genus Aegilops into two sub genera and six sections with eleven diploid and nine polyploid species. Zukovsky (1929, referenced by Kihara 1954) classified eleven diploid and nine polyploid species into nine sections. Bowden (1959) suggested that all diploid species of Aegilops and all allopolyploid species resulting from crosses between Triticum and Aegilops should be transferred into the genus Triticum. He proposed that the taxonomic names be changed in the following manner: A. longissima to T. longissimum. A. squarrosa to T. aegilops, A. bicornis to T. bicorne, and A. speltoides to T. speltoides. In addition, A. squarrosa was often referred to as T. tauschii (see Witcombe 1983 for references). Mac Key (1966) disagreed with this proposal since amphiploids between Triticum and four other genera in the subtribe Triticinae had been produced. The author felt that a better understanding of the species relationships in the *Triticinae* were needed before any major taxonomic changes are made.

The present lack of agreement on the universal acceptance of specific names in *Aegilops* and *Triticum* was illustrated at the Fifth International Wheat Genetics Symposium held in New Delhi in 1978. For instance, Kimber and Hulse (1978) used *T. tauschii* while Nishikawa and Furata (1978) used *A. squarrosa* to describe the same species. Feldman (1978) used *T. longissimum* whereas Nakai (1978) used *A. longissima* to represent the same species.

The taxonomic designations used throughout the text are given in Table I.

Table 1

The super-specific, specific, and sub-specific taxonomic designations for the genus *Triticum* (Mac Key 1966) and *Aegilops* (Witcombe 1983).

Kingdom *Plantae*

Division *Anthophyta*

Class *Monocotyledonae*

Order *Graminales*

Family *Gramineae*

Tribe *Triticeae* Dum.

Subtribe *Triticinae* Holm.

Genus *Triticum* L.

- Section 1. *Monococca* Flaskb.
  - *T. monococcum* L.
    - ssp. *boeoticum* (Boiss.) MK
    - ssp. *monococcum*
  - *T. urartu* Thum.
- Section 2. *Dicoccoidea* Flaskb.
  - *T. timopheevi* Zhuk.
    - ssp. *araraticum* (Jakubz.) MK
    - ssp. *timopheevi*
  - *T. turgidum* (L.) Thell.
    - ssp. *turgidum*
    - ssp. *dicoccoides* (Korn) Thell.
    - ssp. *dicoccum* (Schrank) Thell.
    - ssp. *durum* Desf.
    - ssp. *polonicum* L.
- Section 3. *Speltoidea* Flask.
  - *T. aestivum* (L.) Thell.
    - ssp. *spelta* (L.) Thell.
    - ssp. *vavilova* (Tum.) Sears.
    - ssp. *macha* (Dek. et Men.) MK

ssp. *vulgare* (Vill.) MK

ssp. *compactum* (Host.) MK

ssp. *sphaerococcum* (Perc.) MK

Genus *Aegilops* L.

Section *Amblyoprum*

Species

A. mutica Boiss.

Section *Sitopsis*

Species

A. sharonensis Eig.

A. speltoides var. *speltoides* Tausch
var. *ligustica* (Savign.) Coss.

A. longissima Schweinf. and Muschl.

A. bicornis (Forsk.) Jaub. and Sp.

A. searsii Feld. and Kis.

Section *Vertebrata*

Species

A. squarrosa L.

A. crassa Boiss.

A. vavilovii (Zhuk.) Chenn.

A. ventricosa Tausch

A. juvenalis (Thell.) Eig

Section *Cylindropyrum*

Species

A. caudata L.

A. cylindrica Host

Section *Comopyrum*

A. comosa Sibth, et. Sm.

A. uniaristata Vis.

Section Polyeides

A. ubellulata Zhuk.

A. ovata L.

A. triaristata Willd.

A. columnaris Zhuk.

A. lorentii Hochst.

A. kotschyi Boiss.

A. triuncialis L.

Genome Constitution and Relationships Among the Diploid Tetraploid, and Hexaploid Triticum Species.

Kihara (1924, referenced by Sears, 1948) produced evidence which indicated that the *Triticum* species with 28 and 42 chromosomes are allopolyploids. This conclusion was based on meiotic chromosome pairing analysis in hybrids produced by crossing the diploids with the tetraploids and the tetraploids with the hexaploids. The pollen mother cells (p.m.c.'s) in a pentaploid hybrid produced from a cross between *T. aestivum* and *T. turgidum* had 14^{II} (14 bivalents) and 7^{I} (univalents). Kihara also produced a triploid by crossing *T. turgidum* and *T. monococcum*. Meiotic analysis revealed 7^{II} and 7^{I} in the majority of meiocytes. In this article, the tetraploid species was given the genome formula *AABB* and the hexaploid species was designated AABBDD. The data implied that *T. monococcum* was the A genome donor.

In 1950, Rosenstiel suggested that the tetraploids are autotetraploids and subsequent to their formation, the B genome diverged from the A genome to such an extent that it no longer showed homology with the A genome (referenced in Sears, 1956). Sears (1956) pointed out that if Rosenstiels suggestion were the case, the original tetraploid would have been AAAA. He felt that the probability of seven pairs of chromosomes diverging into the B genome while the other seven pairs remained unchanged is too low to be considered.

Winge's hypothesis of 1917 stated that a polyploid series arises by the hybridization between two parents followed by the doubling of the chromosome number of the hybrid (Watkins 1930). Tetraploid and hexaploid wheats have arisen in this manner

(Kihara, 1924; McFadden and Sears, 1946).

The Source of the A and D Genomes

Although Sax (1922) was the first to report 7^{II} and 7^{I} in a hybrid between T. turgidum and T. monococcum, Kihara (1924) was the first to state that T. monococcum contained the A genome, T. turgidum contained the A and B genomes, and T. aestivum contained the A, B, and D genomes.

In an attempt to identify the D genome donor species, Percival (1921) reasoned that this species should contain morphological characters present in T. aestivum and absent in T. turgidum. These characters are:

"(i) The presence of a single line of long hairs on the summit of longitudinal ridges of the young leaf blades, with shorter ones or none at all on the sides of the ridges.

(ii) Thin-walled, hollow culms.

(iii) The exceptionally tough, non-disarticulating rachis.

(iv) The rounded back and absence of keel on the lower part of the empty glume of a large portion of the race.

(v) The comparatively short awns of the fully bearded ears, and the occurrence of beardless and semi-bearded ears." (Percival, 1921, p. 343)

Aegilops ovata, L., and A. cylindrica, Host., contained these characters and Percival believed that one of these species could have been the D genome donor. However, when Percival (1926) obtained a hybrid between T. aestivum and T. ovata, bivalents were rarely seen. In addition, Sax (1928) observed only univalents in hybrids from a cross between *T*. *turgidum* and *T*. *ovata*. Sax and Sax (1924) obtained hybrids from *T*. *aestivum* and *A*. *cylindrica* ($2n = 4x = 28$). They observed 7^{II} and 21^{I} during meiosis and concluded that *A*. *cylindrica* and *T*. *aestivum* contained one common chromosome set. Gaines and Aase (1926) concluded that the genome in common was the D genome since they observed no bivalent formation in the hybrid when they crossed *T*. *turgidum* with *A*. *cylindrica*. Kihara (1940, referenced by Sarkar and Stebbins, 1956) assigned the genome formula CCDD to *A*. *cylindrica*. This was based on the observation of 7^{II} and 7^{I} in the triploid progeny from a cross between *A*. *caudata* (genome formula CC) and *A*. *cylindrica*.

Pathak (1940) proposed that *A*. *squarrosa* was the D genome donor to common wheat. This proposal was based on chromosome morphology, external plant morphology, growth habit and susceptibility to rust. *T*. *turgidum* possessed one pair of chromosomes with secondary constrictions and one pair with satellites while *T*. *aestivum* contained one pair with secondary constrictions and two pairs with satellites. Referring to the figures given by Senjaninova-Korczagina (1930, referenced by Pathak, ~~1940)~~ *A*. *squarrosa* contained one pair of chromosomes with satellites. In addition, he noted that *A*. *squarrosa* is found growing with wild tetraploid wheats in Turkestan, is susceptible to rusts, and has hollow stems as does *T*. *aestivum*.

Kihara (1944, referenced by Sarkar and Stebbins, 1956) reported the synthesis of a hexaploid hybrid that was morphologically very similar to *T*. *aestivum*. The parents involved were *T*. *turgidum* and *A*. *squarrosa*. The hybrid was obtained by the union of two unreduced gametes. This work was not reported until after the war and in the meantime McFadden and Sears (1946) had produced a hexaploid by crossing *T. turgidum* and *A. squarrosa* and treating the hybrid with colchicine to double the chromosome number. These synthetic hexaploids showed full fertility and could be successfully crossed with *T. aestivum*.

Therefore, by the mid 1940's, *T. monococcum* had been established as the A genome donor to tetraploid and hexaploid wheat while *A. squarrosa* had been established as the D genome donor to hexaploid wheat.

The Source of the B Genome

DNA Studies

In an attempt to identify the B genome donor, Rees (1963) determined the 2C content in *T. monococcum* var. *monococcum*, *T. turgidum var. durum*, *A. squarrosa*, and *T. aestivum*. If little change in nuclear DNA content had occurred since the production of *T. turgidum* and *T. aestivum*, an estimation of the 2C DNA content of the B genome donor could be made. When the DNA contents of *A. bicornis*, *A. longissima*, and *A. speltoides* were determined, Rees (1963) concluded that *A. speltoides* was the most likely contributor of the B genome to tetraploid and hexaploid wheats. Rees and Walters (1965) extended this study and included *T. monococcum* ssp. *boeoticum*, *T. timopheevi*, *T. turgidum* ssp. *dicoccum* and ssp. *dicoccoides*, and *Agropyron triticum* in addition to the species in the Rees (1963) paper. From this study the authors concluded that *T. turgidum* and the subspecies examined had similar DNA contents, *T. turgidum* and *T. timopheevi* had different DNA contents which indicated that the B and G genomes are different. They also concluded that the most likely donor of the B genome is *A. speltoides*.

Subsequent studies have shown that in general, more closely related species share more repeated DNA sequence families than less closely related species (Flavell et. al., 1979). Gerlach and Peacock (1980) mapped the chromosomal locations of highly repeated (C_0t 10^{-2} mol. sec. 1^{-2}) DNA sequences isolated from *T. aestivum* by in situ hybridization of labelled complementary RNA. They found that in *T. aestivum* and *T. turgidum* the label was located on all B genome chromosomes as well as chromosomes 4A and 7A. Each of these labelled chromosomes had a distinctive pattern. From this the authors concluded that the B genome donor species should contain a similar hybridization pattern to that found in *T. aestivum*. Since total highly repeated DNA contained many different families and any one of these families could have diverged during the evolution of species in *Triticum* and *Aegilops*, Peacock et. al. (1981) obtained a single family of repeated sequences isolated by Dennis et. al. (1980) who had shown that this sequence hybridized to the B genome chromosomes of common wheat as well as 4A and 7A. There was minor hybridization to most of the other A chromosomes and 1D, 2D, 3D, and 7D. Peacock et. al. (1981) determined that this family consisted of the triplets $\genfrac{}{}{0pt}{}{\text{GAA}}{\text{CTT}}$ and $\genfrac{}{}{0pt}{}{\text{GAG}}{\text{CTC}}$. When labelled complementary RNA was hybridized to a number of *Triticum* and *Aegilops* species, it was found that *T. urartu*, *T. monococcum* var. *monococcum*, and *A. squarrosa* shows no major hybridization sites while *T. monococcum* var. *boeoticum* has a major site on one chromosome similar to that found on chromosome 7A in *T. turgidum*. There were major sites of hybridization on all chromosomes in *A. longissima*, *A. searsii*, and *A. speltoides*. The authors concluded that the pattern found on the individual chromosomes from *A. longissima* closely resembled the pattern found in the polyploid wheats. It must be noted that none of the above *Aegilops* species had an identical pattern to the B genome of common wheat.

The genes coding for rRNA were found to be located primarily on chromosomes 1B and 6B in *T. aestivum* cv. Chinese Spring (Appels et. al., 1980). Peacock et. al. (1980) compared the Bam H1 and Eco R1 restriction pattern of the 18S and 26S rRNA gene repeating unit in the diploid and polyploid *Triticum* species as well as *A. speltoides*, *A. longissima*, *A. sharonensis*, *A. searsii*, and *A. squarrosa*. All species examined had an identical restriction pattern for the sequences coding for the 18S and 26S rRNA. The spacer region which covers 4.4 kb. exhibited intraspecific as well as interspecific variation and no definite conclusions could be drawn.

The repeating unit coding for 5S rRNA from Chinese Spring shows two distinct size fragments of 420bp and 500bp when Bam H1 restrictred DNA is hybridized to labelled 5S RNA probe. Each of these fragments contain a single 5S RNA gene which is 120 bp. long, and spacer DNA. The 420 bp. repeating unit is located on chromosome 1B. Hybridization experiments by Peacock et. al. (1981) revealed that all tetraploid and hexaploid species of *Triticum* contain a 500bp. and 420 bp. repeat for 5S rRNA, *T. monococcum* contains the 500 bp. repeat as well as some small minor fragments, *A. speltoides* contains the 500 bp. repeating unit, *A. longissima*, *A. sharonensis*, *A. searsii*, and *A. squarrosa* contained a 420 and a 500 bp. repeating unit. Further restriction of the 420 bp. repeating unit with Hae III revealed no variation between these species. The authors concluded that on the basis of the 5S repeating unit, *A. speltoides* could not be the B genome donor to tetraploid and hexaploid wheat.

Nath et. al (1983) hybridized labelled DNA from *T. aestivum* to DNA from a number of *Triticum* and *Aegilops* species and isolated the heteroduplex formed at C_0t 10^4, (this was the point of maximum reassociation). It had been estimated that every 1.5% mismatching of base pairs lowers the thermal stability of the duplex by 1.0°C (Laird et. al., 1969). Nath et. al. reasoned that closely related species should show higher sequence similarities than less closely related species and this relationship should be revealed by heteroduplex stability. This stability could be quantified as the temperature at which 50% disassociation occurs for the heteroduplexes (Tm). By determining the Tm for homoduplex (labelled *T. aestivum* DNA hybridized to unlabelled *T. aestivum* DNA) with that for heteroduplex, Δ Tm was calculated. Labelled DNA from *T. aestivum* was hybridized to unlabelled DNA from *T. urartu*, *A. searsii*, *A. sharonensis*, *A. speltoides*, *A. bicornis*, and *A. longissima*. The Tm was determined for the duplexes formed at C_0t 10^4. The smallest Δ Tm was found in heteroduplex formed between *T. aestivum* and *T. urartu*. However, when Nath et. al. (1984) hybridized labelled DNA's from *T. monococcum* and a synthetic AADD hybrid to *T. urartu* it was found that the A genome was responsible for the homology. *A. searsii* displayed a Δ Tm significantly lower than any of the other species examined and had little homology with A genome or D genome sequences. From this the authors concluded that *A. searsii* was the most likely candidate as the B genome donor.

Meiotic Behavior in Interspecific Hybrids and Amphiploids.

Identification of the B genome donor by a study of the meiotic chromosome behavior of interspecific hybrids is feasible only if the original parental genomes were initially differentiated with respect to pairing affinity and these genomes remained relatively unmodified in the polyploid.

Jenkins (1929) found that 71% of the meiocytes in the hybrid from a cross between T. turgidum (♀) and A. speltoides formed 7^{I} and 7^{II}. The remaining 29% of the meiocytes ranged from $13^{I} + 4^{II}$ to $1^{I} + 10^{II}$. McFadden and Sears (1947) crossed these same species and observed a mean of $7.44^{I} + 4.66^{II}$ with 0-5 multivalents in the hybrid. Riley et. al. (1958) crossed A. speltoides with T. turgidum ssp. turgidum, ssp. dicoccum, and ssp. dicoccoides and observed a mean of $7.92^{I} + 5.94^{II} + 0.40^{III}$, $6.70^{I} + 6.22^{II} + 0.62^{III}$, and $5.94^{I} + 5.21^{II} + 1.59^{III}$ respectively in the hybrids. Since the A genome donor to T. turgidum was T. monococcum and Sears (1956a) reported a mean of $8.6^{I} + 2.7^{II} + 0.15^{III}$ in a hybrid produced by crossing T. monococcum and A. speltoides, it was concluded that the increase in pairing in the triploid hybrid produced by crossing T. turgidum with A. speltoides was due to B genome homology. Riley et. al. (1958) also crossed T. monococcum and A. speltoides and observed a mean of 3.37^{II} in the hybrid. In addition, these authors crossed T. turgidum ssp. dicoccoides with A. bicornis, and T. turgidum ssp. turgidum with A. sharonensis, and T. turgidum ssp. dicoccum with A. longissima and reported the mean number of bivalents in the hybrids were 1.78 ± 0.18, 2.22 ± 0.15, and 1.60 ± 0.17 respectively. The authors concluded that on the basis of meiotic pairing data, A. sharonensis, A. bicornis, and A. longissima could not be the B genome donor to tetraploid wheat. However, Kihara (1949) reported a mode of 7^{II} in the hybrid from a cross between A. speltoides and A. longissima while Kimber (1961, referenced by Kimber, 1974a) observed a mean of 6.7^{II} in the hybrid from a cross between A. speltoides and A. longissima, and 6.86^{II} in the hybrid resulting from a cross between A. bicornis and A. longissima. On the basis of this data, Kimber (1961) concluded that A. speltoides, A. longissima, and A. bicornis share a high degree of homology.

Sears and Okamoto (1958) found that when chromosome 5 (belonging to the B genome, Okamoto, 1957) was missing in T. aestivum, homoeologous pairing occurred. They found that when T. aestivum that was monosomic for chromosome 5B was crossed to T. monococcum, $2\text{-}7^{II}$ were observed when 5B was present and $5\text{-}13^{II}$ were observed in its absence. At the same time, Riley (1958) reported a haploid line of T. aestivum with only 20 chromosomes. At meiosis, a mean of $4.2^{II} + 0.8^{III}$ were observed and 29% of the cells had 5-7 bivalents. Riley et. al. (1960) determined that this line lacked chromosome 5B. Using ditelocentric lines, the gene(s) responsible for this behavior was found to be located on the long arm of this chromosome (Riley, 1960).

Riley et. al. (1961) crossed A. speltoides and A. longissima with T. aestivum that was monosomic for chromosome 5B. Hybrids produced with A. longissima had a mean of $9^{I} + 7^{II} + .75^{III} + .5^{IV}$ when chromosome 5B was absent and $24^{I} + 2^{II}$ when 5B was present while hybrids produced with A. speltoides had a mean of $6^{I} + 6^{II} + 2^{III} + 1^{IV}$ whether or not chromosome 5B was present. From this data, it was concluded that A. speltoides contained a genotype that would override the meiotic property of strict homologous pairing in the polyploid wheats.

Kushnir and Halloran (1981) crossed *A. sharonensis* ♀ with *T. turgidum* ssp. *dicoccoides*. The hybrid had a mean of 2.23^{II} and an amphiploid was produced which had 73% normal pollen and seed set was 43.4%. They concluded that *A. sharonensis* had a cytoplasm compatible with *T. turgidum*. The authors crossed ♀ *A. sharonensis* with *T. monococcum* and observed of 0.3^{II} in the hybrid. This value fell between a mean of 0.9^{II} found in a haploid line of *T. aestivum* (Kimber 1961, referenced in Kimber 1974a) and 0.18^{II} found in a haploid line of *T. turgidum* (Kimber et. al. 1978). The data indicated that there was no homology between the chromosomes of *A. sharonensis* and *T. monococcum*. After treatment of the *A. sharonensis* x *T. monococcum* hybrid with colchicine, a mean of 13.85^{II} were observed in the amphiploid. A hybrid between *T. turgidum* and the amphiploid was produced and a mean of 8.11^{II} was observed. This value was similar to 8.05^{II} observed in a hybrid between *T. turgidum* and an amphiploid produced by colchicine treatment of a *A. bicornis* x *T. monococcum* hybrid (Sears 1956a) and higher than 7^{II} observed in a hybrid between *T. turgidum* and *A. longissima* x *T. monococcum* amphiploid (Tanaka, 1956). Kushnir and Halloran (1981) crossed *T. aestivum* that was homozygous for the ph$_{1b}$ allele and therefore allowed homoeologous pairing, with *A. sharonensis*. Since some divergence must have occurred since the hybridization events leading to the production of tetraploid and hexaploid wheats, they felt that with the Ph allele present, pairing would be suppressed. They observed a mean of 9.66^{II} (range $7\text{-}12^{II}$) in the hybrid. This value is higher than 7.58^{II}

obtained when nulli 5B *T. aestivum* was crossed with *A. longissima* and 5.23^{II} observed when nulli 5B *T. aestivum* was crossed with *A. speltoides* (Riley and Law 1965). To estimate the number of bivalents that were due to B genome chromosomes pairing with *A. sharonensis* chromosomes, they subtracted 3.82^{II} which were observed in nulli 5B haploid lines of *T. aestivum* (Riley and Law, 1965) since this was due to homoeologous pairing. In addition, since 0.3^{II} were observed in the *T. monococcum* x *A. sharonensis* hybrid and it was assumed that *A. sharonensis* and the D genome would have the same affinity, 0.6^{II} was subtracted from this total. They calculated that 5.24^{II} per cell were due to the B genome chromosomes pairing with *A. sharonensis*. The authors felt that this value was very close to the value which implicated *T. monococcum* as the A genome donor to the polyploid wheats and therefore concluded that *A. sharonensis* was a likely candidate as the B genome donor to tetraploid and hexaploid wheats.

Johnson (1975) crossed *T. monococcum* with *T. urartu* and observed 7^{II} in the sterile hybrid. An amphiploid produced from this hybrid had $12^{II} + 1^{IV}$ or $10^{II} + 2^{IV}$. The triploid hybrid obtained from a cross between *T. turgidum* and *T. urartu* had $7^{I} + 7^{II}$ or $9^{I} + 6^{II}$. To determine whether the chromosomes of *T. urartu* were paired with the A or the B genome chromosomes, Dvořák (1976) crossed *T. urartu* with lines of *T. aestivum* that were ditelosomic for the A and B chromosomes. Chapman et. al. (1976) crossed *T. urartu* with lines of *T. aestivum* that were ditelosomic or doubly ditelosomic for the A and B chromosomes. The results were essentially the same . *T. urartu* paired with the A genome chromosomes of common wheat except for chromosome 4A. Dvořák (1976) reported that when 4Aα (where α indicated that it was not known which of the two arms of chromosome 4A was present) was telocentric, heteromorphic bivalents were not observed. Chapman et. al. (1976) found that when both arms of chromosome 4A were present as telocentrics, no heteromorphic bivalents were observed but when 4Aα was telocentric, heteromorphic bivalents were observed. The authors concluded that on the basis of telocentric pairing data, the chromosomes of *T. urartu* resembled the chromosomes of the A genome.

The pairing data from various diploid, triploid, and tetraploid hybrids indicated that the chromosomes in *Triticum* and *Aegilops* species retained some degree of homoeology since their divergence from some pivotal genome. It was possible that when the tetraploid species was produced, the chromosomes from the A and B genome parents had a high degree of homoeology such that chromosomes from both parents, without the Ph allele, could pair with each other (Johnson and Dhaliwal 1978). Johnson and Dhaliwal (1978) claimed that subsequent to hybridization, one of the two homoeologues diverged such that one genome is now made up of chromosomes from both parents and have diverged to a point where they fail to pair with either parent and the other genome has diverged little and retains the ability to pair with both parents. This reasoning evolved from the observation that *T. monococcum* x *T. urartu* hybrids had 6.97^{II} yet were completely sterile. Dhaliwal and Johnson (1982) crossed an amphiploid produced from a *T. monococcum* x *T. urartu* hybrid with *T. aestivum* that was monosomic for chromosome 5B. In the progeny where 5B was present, $11.06^{I} + 3.74^{II} + 3.13^{III}$ were observed whereas when 5B was absent, $2.09^{I} + 6.08^{II} + 3.07^{III} + 0.82^{IV} + 0.09^{V}$ were observed. The authors concluded that when the

Ph gene was absent, an increase in bivalent and quadrivalent associations indicated that the A and B chromosomes from *T. turgidum* were pairing with *T. monococcum* and *T. urartu*, respectively.

Somatic Chromosome Morphology

Attempts have been made to determine the B genome donor species on the basis of somatic chromosome morphology. As was noted earlier, Pathak (1940) concluded that on the basis of chromosome morphology *A. squarrossa* was the D genome donor to common wheat.

Pathak (1940) observed two pairs of chromosomes with large satellites in *T. turgidum*. Using monosomic lines of *T. aestivum*, Morrison (1953) concluded that these chromosomes belonged to the B genome. Riley et. al. (1958) compared the chromosomes with satellites found in *A. speltoides*, *A. bicornis*, *A. sharonensis*, and *A. longissima* with those in *T. turgidum*. They concluded that *A. speltoides* contained satellited chromosomes morphologically similar to those found in *T. turgidum*. In a series of papers, Giorgi and Bozzini (1969 a, b, c) described detailed measurements of the somatic chromosomes from eight subspecies of *T. turgidum*, *T. monococcum* ssp. *boeoticum*, *T. urartu*, *A. bicornis*, *A. speltoides*, and an *A. speltoides* x *T. monococcum* amphiploid. For the chromosomes with satellites (Sat), the satellite length was omitted. Median chromosomes (M) were defined as chromosomes with an arm ratio between 1 and 1.25, submedian chromosomes (Sm) had an arm ratio between 1.26 and 1.75, and subterminal chromosomes (St) had an arm ratio of 1.76 or greater. *T. turgidum* was reported to contain 2 Sat, 2 St, 7 Sm, and 3 M pairs of chromosomes. Whereas Riley et. al. (1958) reported one pair of chromosomes with small satellites in *T.*

monococcum, Giorgi and Bozzini (1969b) reported two pairs of chromosomes with small satellites in T. monococcum and T. urartu. The latter authors report that T. monococcum and T. urartu are karyotypically very similar and both contain 2 Sat, 3 Sm, and 2 M chromosomes. Giorgi and Bozzini (1969b) observed that A. speltoides contain 2 Sat, 1 St, 2 Sm, and 2 M pairs of chromosomes while A. bicornis contained 2 Sat, 4 Sm, and 1 M pairs of chromosomes. They found no direct correlation when comparing the morphology of the chromosomes in T. monococcum, A. bicornis or A. speltoides with the chromosomes from T. turgidum.

Feldman (1978) karyotyped A. searsii and found it to contain 2 Sat, 3 Sm, and 2 M pairs of chromosomes. Measurements of the arm lengths or total chromosome length were not made.

Kushnir and Halloran (1981) used the data of Giorgi and Bozzini (1969 a, b, c) to determine the morphology of the B genome in tetraploid wheat. The satellited chromosomes from T. monococcum were not visible in T. turgidum. Since Sat 1 from T. monococcum had an arm ratio of 1.95, it must be one of the two St chromosomes in T. turgidum. This is in line with the data from Sears (1954) who determined that chromosome 1A in T. aestivum had an arm ratio of 1.91. Kushnir and Halloran (1981) concluded that the B genome donor possessed 2 pairs of sat chromosomes with large satellites, 1 St pair, 3 pairs of Sm, and 1 pair of M chromosomes. On the basis of chromosome morphology, the authors concluded the A. sharonensis could have been the B genome donor to tetraploid wheat.

Waines and Kimber (1973) karyotyped 10 wild biotypes collected from Europe and Iran, and 4 cultivars of T. monococcum. They found a variety of intraspecific variants with respect to the satellited chromosomes. In some lines, only one satellited pair of chromosomes was visible, in other lines two pairs of chromosomes with small satellites were visible, and in other lines the satellite size varied. They suggested that caution be exercised when trying to relate different species on the basis of karyotype analysis only.

Cytoplasmic Studies

Previous studies had indicated that cytoplasmic divergence had occurred in *Triticum* and *Aegilops*. Kihara (1951) noticed that the degree of vigour of a hybrid resulting from an intergeneric cross between *Triticum* and *Aegilops* depended on the direction of the cross (that is, which parent was used as the pollen donor). Although all crosses have not been made between *Aegilops* and *Triticum*, Tsunewaki et. al. (1976) classified 23 cytoplasms into eight plasma types. This was based on the phenotypes of alloplasmic lines. An alloplasmic line is produced by crossing a species, for example ♀ *A. sharonensis* with *T. aestivum* and backcrossing the progeny to ♂ *T. aestivum*. In this way, lines are produced that contain the nuclear genome of *T. aestivum* and the cytoplasmic component of *A. sharonensis*

Since the cytoplasmic component is transmitted by the maternal parent only, Chen, et. al. (1975) determined the isoelectric points of the polypeptides that make up the large and small subunits of ribulose -1, 5, -biphosphate carboxylase-oxygenase (rubisco). Rubisco catalyzes CO_2 fixation during photosynthesis and CO_2 emission during photorespiration and is composed of two subunits. The small subunit is composed of two polypeptide chains and is coded for in the nuclear genome. The large subunit was found to consist of three polypeptide chains that are encoded in the chloroplast genome (Kawashima and Wildman 1972; Chan and Wildman 1972; Hartley, et. al. 1975; Coen et. al. 1977). The objectives of Chen, et. al. (1975) were to establish the maternal parents of *T. turgidum* and *T. aestivum*. In all species studied, the small subunit had an identical isoelectric point. Isoelectric focusing of the polypeptides that make up the large subunit revealed two patterns. *T. aestivum*, *T. turgidum*, and *A. speltoides* had a higher isoelectric point than *T. monococcum*, *T. urartu*, and *A. squarrosa*. From this data, the authors concluded that the female parent involved in the production of *T. turgidum* could not have been *T. monococcum*. Similarly, the female parent involved in the production of *T. aestivum* must have been *T. turgidum* and not *A. squarrosa*. In addition, it was concluded that *T. urartu* could not be the B genome donor to tetraploid and therefore hexaploid wheats. Although an identical isoelectric point does not indicate an identical amino acid sequence, the data suggested that *A. speltoides*, or another species containing an identical subunit pattern could have been the female parent that donated the B genome to tetraploid and hexaploid wheats.

Johnson (1976) argued that since the above data was obtained from a single accession in each species, intraspecific variation could occur in the large subunit of rubisco. However, Gray et. al (1976) examined the isoelectric point of the polypeptides in rubisco from 20 cultivars of *Nicotiana tabacum* and 10 cultivars of N. suaveolens obtained from breeders located in different parts of the world. They found no intraspecific variation. Each species had a single pattern.

Hirai and Tsunewaki (1981) extended this study using alloplasmic lines of T. aestivum cv. Chinese Spring. Their results were in agreement with Chen, et. al. (1975). In addition they found that A. longissima contained a rubisco large subunit that comigrated with A. speltoides. A. sharonensis and A. bicornis contained a rubisco large subunit that comigrates with T. monococcum, T. urartu, and A. squarrosa. The authors concluded that A. longissima as well as A. speltoides could have been the B genome donor to T. turgidum and T. aestivum.

Using a variety of restriction enzymes, the restriction fragment pattern of chloroplast DNA in many diploid and tetraploid Triticum and Aegilops species have been reported (Vedel, et. al. 1976; Vedel et. al. 1978; Ogihara and Tsunewaki 1982; Tsunewaki and Ogihara 1983).

Tsunewaki and Ogihara (1983) used the restriction enzymes Bam Hl, EcoRl, Hind III, Kpn I, Pst I, Sma I, and Xho I. Compared to the fragment pattern of T. aestivum and T. turgidum, T. monococcum differed by 11 fragments, T. urartu by 8 fragments (digestion with Sma I and Xho I was not completed), A. speltoides by 10 fragments, A. bicornis by 10 fragments, and A. searsii by 10 fragments. The fragment pattern obtained from A. longissima was identical to T. turgidum and T. aestivum.

Protein Studies

In a series of papers, B.L. Johnson isolated alcohol soluble endosperm proteins from a number of Triticum and Aegilops species and determined the electrophoretic patterns using a polyacrylamide gel. He reasoned that if each genome has equal expression, the pattern found in a hybrid should reflect its parents band profile. In this way, Johnson (1967) found that A. cylindrica had an electrophoretic pattern identical to that produced when equimolar amounts of alcohol soluble endosperm isolated from A. squarrosa and A. caudata were mixed. This evidence supported the meiotic pairing data obtained by Kihara (1954), who crossed A. cylindrica with A. caudata and found primarily 7^{II} and 7^{I} at meiosis in the hybrid. Similarly, when A. cylindrica was crossed with A. squarrosa, 7^{II} and 7^{I} were observed in most of the meiocytes from the hybrid. A. cylindrica (2n = 4x = 28) was given the genome formula CCDD, A. squarrosa (2n = 2x = 14) was given DD, and A. caudata (2n = 2x = 14) was given the formula CC.

The electrophoretic pattern of alcohol soluble endosperm proteins have been determined for T. aestivum, T. turgidum, and T. monococcum (Johnson and Hall, 1965; Johnson, 1972a); T. timopheevi (Johnson, 1967a); A. squarrosa (Johnson, 1967b); A. speltoides, A. longissima, and A. sharonensis (Johnson, 1972b). From these studies, Johnson (1972b) concluded that none of the above mentioned Aegilops species could have been the B genome donor to T. turgidum and therefore T. aestivum.

Johnson (1975) determined the electrophoretic profile of alcohol soluble endosperm proteins from 534 accessions obtained from 230 collection sites, and representing all distinguishable morphological types of *T. turgidum*, *T. timopheevi* and *T. monococcum*. Also, in this paper, he determined the electrophoretic pattern of the water soluble albumins from equimolar extracts of selected parents. He found that mixing water soluble endosperm proteins from accessions of *T. monococcum* and *T. urartu* obtained from Lebanon accounted for all the albumin bands of *T. turgidum*. The mixture, however showed one additional band. From this Johnson concluded that *T. urartu* could be the B genome donor to common wheat.

Purothionins

Unless otherwise stated, all work referred to was completed using commercial varieties of *T. aestivum*.

While studying the components of wheat flour and their effects on baking quality, Balls and Hale (1940) isolated a crude lipoidal extract that was subsequently purified (Balls et. al. 1942a) and called lipopurothionin (Balls et. al. 1942b). The above extraction procedure used petroleum ether. When dilute acid was used free proteins were extracted and these were called purothionins. Balls et. al (1942a) determined that this protein contained 20% arginine and 16% cystine. These values are unusually high since the average values for wheat endosperm protein are 4% arginine and 2% cystine (Fisher et. al. 1968). Nimmo et. al (1968) found two overlapping bands when extracted purothionin was subjected to polyacrylamide gel electrophoresis. Redman and Fisher (1968) isolated these two fractions using ion exchange chromatography and the fraction that eluted first was named β-purothionin while the fraction that

eluted last was named α-purothionin. Circular dichroism data and intrinsic viscosity values indicated that α- and β-purothionins form compact globular structures in water with 40% α-helical structures (Nimmo et. al. 1974). Ohtani et. al. (1975) determined the amino acid sequence of two proteins that they called "purothionin A-I" and "purothionin A-II" and found them to be 45 amino acids long. Mak and Jones (1976a) found that the amino acid sequence of β-purothionin was identical to "purothionin A-I". Using a more efficient separation procedure, Jones and Mak (1977) determined that the α-purothionin fraction was made up of two distinct components that they termed α_1- and α_2-purothionins. These authors found that the amino acid sequence of α_1-purothionin was identical to that of "purothionin A-II". Jones et. al. (1982) isolated two forms of purothionins from _T. turgidum_ ssp. _durum_. One form had an amino acid sequence identical to α_1-purothionin
sequence identical to β-purothionin. Jones and Mak (1983) reported that _T. monococcum_ contained only β-purothionin and Jones et. al. (1983) determined that _A. squarrosa_ contained α_2-purothionin. Since the A genome donor to tetraploid and hexaploid wheat contained β-purothionin and the D genome donor to _T. aestivum_ contained α_2-purothionin, it was reasoned that the B genome donor should contain α_1-purothionin (Jones et. al. 1982). Carbonero and Garcia-Olmedo (1969) used starch gel electrophoresis to determine that _A. speltoides_, _A. longissima_, and _A. bicornis_ contained purothionins of the α-type. However, this type of analysis was not sensitive enough to detect sequence identity to the α_1- or α_2-purothionin.

Function

Stuart and Harris (1942) demonstrated that crude purothionin extract had bactericidal and fungicidal properties. In vitro activity was greatest against gram positive bacteria but lethal affects toward some gram negative bacteria were also observed. Fungicidal activity against Saccharomyces cerevisea, Debaromyces nadiformes, and Endomycopsis albicans was observed, however the mycelial fungi tested were resistant. When crude purothionin was injected intraperitoneally into mice, no in vivo activity against pneumococci or streptococci was observed. Coulson et. al. (1942) found that high concentrations of purothionin were toxic to mice when injected intraperitoneally or intravenously, but no affect was seen when the same amount was orally injected. These authors also observed that isolated uteri from virgin guinea pigs were induced to contract upon treated with purothionin. Okada et. al. (1970) reported that the toxicity of purothionins towards S. cerevisea could be neutralized by the addition of Ca^{2+}, Zn^{2+}, or Fe^{2+}. Fernandes de Caleya et. al. (1972) tested crude purothionin against various phytopathogenic bacteria and observed that all gram positive and some gram negative species were sensitive. Hernandez-Lucas et. al. (1974) determined that purified purothionin had lethal affects toward S. uvarum (syn. carlsbergensis) and S. cerevisea. Okada and Yoshizumi (1973) reported that purothionin inhibited respiration and fermentation in S. uvarum. The toxin appeared to bind to the cell membrane and caused leakage of potassium and phosphate ions, proteins, and nucleotides. The addition of Ca^{2+} ions did not prevent binding of the toxin to the cell membrane but did prevent cytoplasmic leakage and cell death. The authors concluded that the mode of action of this toxin is to bind to the cell surface and then to change the membrane permeability. Nakanishi et. al. (1979) studied the effects of purothionin on mammalian cell lines. The authors found that the toxic effect was limited to actively growing cell lines. To pinpoint the exact stage in the cell cycle where the toxin was acting, the authors synchronized cultures of Balb/3T3 cells and compared the time when the lethal effects were observed to the time of DNA synthesis. The authors concluded that purothionin selectively kills cells at S-phase. These conclusions were supported by the observation that contact inhibited baby hamster kidney (BHK) cell lines showed no lethal effect to purothionin while BHK lines transformed by polyoma virus were sensitive to the toxin. Kramer et. al. (1979) were the first to report purothionin toxicity toward invertebrates. Injections of purothionin into fourth instar larvae of the tobacco hornworm (Manduca sexta) resulted in death, whereas oral administration had no affect. The authors also reported that bathing the adult Manduca flight muscle fibre with various concentrations of purothionin disrupted the membrane potential in a dose dependent manner. Reduced and alkylated protein had no affect. The authors concluded that purothionin binds to the cell membrane and induces changes in the membrane permeability. Carrasco et. al. (1981) observed that purothionins inhibited transcription, translation, and DNA synthesis cultured in baby hamster kidney cell lines, monkey CV1 cells, mouse L cells, and human He La cells. These affects were attributed to changes in cell membrane permeability since leakage of nucleotides were observed. In addition, at low purothionin concentrations which do not disrupt translation, it was observed that antibodies which normally could not transverse the cell membrane could then inhibit translation.

While the above observations indicate that purothionins could have a role in protecting the seed from predator invasion, there is no proof that this is the *in vivo* function of these proteins.

Homologous proteins have been extracted from the European miseltoe (*Viscum album*) and related species (Samuelsson, 1973), *Hordeum vulgare* L. (Redman and Fisher, 1969), *Secale cereale* (Hernandez Lucas et. al. 1978), and the *Crambe abyssinica* (Van Etten et. al. 1965).

Using compensating nulli-tetrasomic lines and ditelosomic lines of *T. aestivum*, Fernandez de Caleya et. al. (1976) determined that the gene coding for β-purothionin is located on the long arm of chromosome 1A while the genes coding for the α-purothionins were located on the long arm of chromosome 1B and 1D.

MATERIALS AND METHODS

Seed Source

Seed was kindly provided by the United States Department of Agriculture, Betsville, Maryland; Agriculture Canada, Ottawa, Ontario; Dr. B.L. Johnson, University of California, Riverside; and the Department of Plant Science, University of Alberta, Edmonton, Alberta. A list of the species, line number, and source is given in Table II.

An example of a representive head from each species is given in Plate 1. To confirm the identity of each species, samples of each species were sent to Dr. B.L. Johnson at the University of California, Riverside, and Dr. B.S. Gill at Kansas State University.

An example of a representative spikelet from each species is given in plate 2. Some of the morphological characteristics are given in Table 3. The geographical location of wild *Triticum* and *Aegilops* species are given in Table 4.

There was some difficulty in growing the putative B genome donors at this latitude. The species were seeded indoors in February and transplanted to the field in the middle of May. *A. speltoides* and *T. urartu* would usually be ready for harvest at the end of September. All other species had to be transferred to growth chambers or green houses and would be ready for harvest by December or January.

Species	Accession Number	Source
T. turgidum	CV Stewart	Department of Plant Science University of Alberta, Edmonton, Alberta Canada
T. monococcum	PI 290 509	USDA, Beltsville, Maryland, U.S.A.
A. speltoides	G 1062	B.L. Johnson, Department of Botany and Plant Science, University of California, Riverside, California
A. longissima	PGR 8714	The Plant Gene Resources of Canada, Agriculture Canada, Ottawa, Ontario
A. bicornis	CI 32	USDA, Beltsville, Maryland, U.S.A.
A. searsii	PGR 7808	The Plant Gene Resources of Canada, Agriculture Canada, Ottawa, Ontario
T. urartu	PGR 6150	The Plant Gene Resources of Canada, Agriculture Canada, Ottawa, Ontario
A. sharonensis	PGR 8731	The Plant Gene Resources of Canada, Agriculture Canada, Ottawa, Ontario

Table 2. The accession number and source of each species used in this study.

PLATE 1. A representative head sample from:

1) _T._ _turgidum_ var. Stewart
2) _T._ _monococcum_
3) _A._ _speltoides_
4) _A._ _longissima_
5) _A._ _searsii_
6) _A._ _bicornis_
7) _T._ _urartu_
8) _A._ _sharonensis_

½ actual size

Plate 2. A representative lateral spikelet and seed sample from:

1 - *T. turgidum*
2 - *T. monococcum*
3 - *A. speltoides*
4 - *A. longissima*
5 - *A. bicornis*
6 - *A. searsii*
7 - *T. urartu*
8 - *A. sharonensis*

	1	2	3	4	5	6
Spike length (cm)	13-15	14-16	5-7	10-12	8-10	7-9
Number of spikelets per spike	12-14	13-15	17-19	11-13	14-16	18-20
Number of seeds per spikelet	2	2	1 or 2	2	2	2
Free caryopsis	-	-	+	+	+	-
spike disarticulates into single spikelets	-	+	+	-	+	+
Awns on terminal spikelet	+	+	+	+	+	+
Awns on lateral spikelet	-	-	+	-	+	+

Table 3. Morphological characteristics of representative heads from 1 - A. speltoides, 2 - A. longissima, 3 - A. bicornis, 4 - A. searsii, 5 - T. urartu, 6 - A. sharonensis, + Yes
- No

Species	Distribution
T. turgidum	Israel, Jordan, Syria, Iran, Iraq, Turkey
T. monococcum	Widely spread over western Asia
A. speltoides	N. Israel, Syria, S. Turkey, N. Iraq, W. Iran
A. longissima	Israel, Jordan, S. Syria, Egypt
A. bicornis	S. Israel, S. Egypt, Lybia
A. searsii	Israel, Jordan, Lebanon, Syria
T. urartu	Lebanon, Syria, S.W. Turkey, Iran, Iraq, U.S.S.R.
A. sharonensis	Israel

Table 4. The geographic distribution of wild *Triticum* and *Aegilops* species. Taken from Witcombe (1983), Feldman and Kislev (1977), Johnson (1972).

Purothionin Extraction

All seeds were milled in a Udy Cyclone Mill and the flour passed through a 0.4 mm mesh screen. The quantities of flour obtained were 50g from A. speltoides, 75g from A. longissima, 47g from A. searsii, 45g from A. bicornis, 40g from A. sharonensis, and 65g from T. urartu.

The flour was mixed with 400 ml of 0.05 M H_2SO_4 at 4°C and stirred at 15 minute intervals for one hour. Solutions from A. speltoides and A. longissima were centrifuged at 5,000 rpm for ten minutes at 4°C, A. searsii and A. bicornis were centrifuged at 7,000 rpm for ten minutes at 4°C, and A. sharonensis and T. urartu were centrifuged at 10,000 rpm for 15 minutes at 4°C in a Beckman J21C Centrifuge, R-14 rotor. The procedure to this point was similar to that used by Okada et. al. (1970). The supernatents were poured off and kept at 4°C. The pellets were suspended in 200 ml (A. speltoides, A. longissima) or 250 ml (A. searsii, A. bicornis) of 4°C 0.05 M H_2SO_4 and allowed to stand in an ice bath for 30 minutes. The suspensions were centrifuged at 7,000 rpm for ten minutes at 4°C. The supernatents from each species were individually pooled and adjusted to pH 8.6 with 4N NaOH. These solutions were allowed to stand in an ice bath for one hour. If the solutions turned cloudy, the sample was centrifuged. Solutions from A. longissima, A. searsii, A. bicornis, A. sharonensis, and T. urartu were centrifuged at 9,000 rpm for ten minutes at 4°C.

The clear supernatents were adjusted to pH 5.2 with acetic acid and allowed to reach room temperature. Each solution was gravity loaded onto a 1.2 x 15 cm carboxymethyl cellulose (CMC, Whatman

CM52 preswollen microgranular) column that had been equilibrated with 3 volumes of 0.3 M ammonium acetate (NH_4Ac), pH 5.2. After loading, the columns were washed with 0.3 M NH_4Ac until a baseline absorbance value was reached, indicating no further 280 nm absorbing material was being eluted from the column.

Linear gradients were developed through the columns. A 0.5-1.0 M (250 ml. of each concentration) NH_4Ac, pH 5.2, gradient was developed through the column containing *A. speltoides* and *A. longissima* protein, a 0.3-1.1 M (250 ml of each concentration) gradient was developed through the columns that contained *A. searsii* and *A. bicornis* protein and a 0.4-1.2 M (250 ml of each concentration) gradient was developed through the columns that contained *A. sharonensis* and *T. urartu* protein. The column effluent was monitored by absorbance at 280 nm, with an Isco UA5 uv monitor.

It has been observed that purothionins were precipitated in the presence of 10% SDS (B.L. Jones, personal comm.). In all species tested, positive results occurred with the faction that eluted last.

The appropriate fractions from *A. speltoides* , *A. longissima*, *A. searsii*, and *A. bicornis* were collected, diluted with 1 volume of distilled and deionized water, and loaded onto a 1.2 x 15 cm CMC column that had been equilibrated with 3 volumes of 0.05 M NH_4Ac, pH 5.2, gradient was developed through the column and purification was indicated by a symmetrical peak. The appropriate fractions were collected and freeze dried twice to remove the NH_4Ac.

The appropriate fractions from *T. urartu* and *A. sharonensis* were loaded onto a C-18 reverse phase column (Beckman, 5 cm x 4.6 mm) and eluted with a trifluoroacetic acid/acetonicrile gradient (see Table 5 for composition). Native purothionins elute between 9 and 10 minutes (B.L. Jones, personal comm.).

Reduction and Pyridylethylation of Purothionins

Samples of the purothionins were denatured with 0.5 M Tris buffer, pH 7.5, that contained 3 M deionized urea, and flushed with nitrogen gas for 2 minutes. To reduce the disulfide bonds, 2-mercaptoethanol (Pierce Chemical Co.) was added and the solution was flushed with N_2(g) for 10 seconds and sealed. The mixture was incubated at 50°C for 30 minutes in a water bath. Four-vinyl pyridine was added to the solution, purged with N_2(g) for 15 seconds, sealed, and incubated at 50°C for 30 minutes (see Table 6 for components). The samples from *A. bicornis*, *A. searsii*, *A. longissima*, and *A. speltoides* were loaded onto a 1.2 x15 cm. CMC column that had been equilibrated with 3 volumes of 0.5 M NH_4Ac. A 0.5-1.0 M (250 ml of each) NH_4Ac, pH 5.2, gradient was developed through the column and the eluates were monitored at 280 nm. The appropriate fractions were collected and twice freeze dried. This procedure was modified from Mak and Jones (1976b). The samples of reduced and pyridylethylated purothionin from *A. sharonensis* and *T. urartu* loaded onto C-18 columns and fractions that eluted between 7.5 and 8.0 minutes were analyzed.

Time	% 0.3% TFA	%CH_3CN
0	10	90
9	40	60
12	10	90

Table 5. Gradient composition used in the HPLC separation of purothionin from the crude protein mixture.

Species	Mg. of Purothionin	Tris-Urea (μl)	2-Mercaptoethanol (μl)	4-Vinyl Pyridine (μl)	Reduced and Pyridylethylated Protein Recovered (mg)
A. speltoides	5.1	500	11.3	14.2	3.5
A. longissima	2.1	500	27.0	40.0	1.3
A. searsii	2.3	500	12.0	15.0	1.1
A. bicornis	1.7	500	12.0	15.0	0.9
A. sharonensis	.8	500	10.0	13.0	0.4
T. urartu	2.0	50	10.0	13.0	1.5

Table 6. Amounts of each component used in the reduction and pyridylethylation of purothionin.

Peptide Production

Peptides were produced by treating the reduced and pyridylethylated protein with three site specific proteolytic enzymes.

(A) Clostripain hydrolysis

Clostripain, under optimal conditions, cleaves peptide bonds in which the carboxyl group is contributed by arginine (Jones and Lookhard 1983).

Purothionin (0.2 mg) was dissolved in 50 µl of 20 mM Tris HCl, pH 7.4, buffer. The enzyme solution consisted of 90 µg of Clostripain (Boehringer-Mannheim), 73 mg of $CaCl_2$, and 0.8 mg of dithiothretol which was dissolved in 2.0 ml of distilled and deionized water. Fifty µl of enzyme solution was added to the protein buffer solution, mixed well, and incubated at 39.5°C. Fifty µl were removed at 15 and 30 minutes. The samples were frozen to stop the reaction and subsequently dried under vacuum.

(B) Trypsin hydrolysis

Trypsin cleaves peptide bonds in which the carboxyl group is contributed by lysine or arginine. When lysine is the amino terminal residue, it is not cleaved (Jones and Lookhart 1983).

Purothionin (0.2 mg) was dissolved in 100 µl of 0.1 M PCPES (piperazine-N_2N^1-bis [2-ethane sulfonic acid], Sigma, adjusted to pH 6.8 with NaOH). The enzyme solution consisted of 0.1 mg of trypsin (Worthington, TOCK treated) per ml of PIPES buffer. Twenty µl of enzyme was added to the protein buffer solution, mixed well, and incubated at 30°C for one hour. The reaction was terminated by freezing and the samples were dried.

(C) Endoproteinase Lysine-C (EPLC) hydrolysis

EPLC cleaves peptide bonds in which the carboxyl group is contributed by lysine (Jones and Lookhart 1983).

Purothionin (0.1 mg) was dissolved in 100 µl of buffer (0.025 M Tris and 0.001 M EDTA that was adjusted to pH 7.7 with HCl). Five µl of EPLC solution (consisting of 1 µg of EPLC [Boehringer-Mannheim] in 5 µl of distilled and deionized H_2O) was added to the protein buffer solution, mixed well, and incubated at 30°C for 4 hours. One half of the sample was removed at 1 hour. The reaction was terminated by freezing and the samples were dried under vacuum.

Reduced and pyridylethylated purothionin from T. urartu and A. sharonensis (0.1 mg) was dissolved in 400 µl of 0.1 M ammonium bicarbonate, pH 8.0. Five µl of EPLC solution was added to the protein buffer solution and incubated for 75 minutes at 37°C. One half of the sample was removed at 45 minutes. The reaction was terminated by freezing and the samples were dried under vacuum.

The elution gradient was mixed and injected using a Varian 5060 pump. The eluate was pumped through two uv detectors in series which enabled recording at 220 nm with a Tracor 970 variable wavelength detector and at 280 nm with a Isco UA5 detector. Hewlett-Packard 3388A and 3385A automation systems were used to record and analyze the outputs from the detectors. A 0.1% TFA-acetonitrile gradient was used to elute the peptides (see Table 7 for gradient composition). The separated peptides were collected and freeze dried. This procedure was modified from Jones and Lookhart (1983).

The peptides generated by EPLC digestion of purothionin isolated from T. urartu and A. sharonensis were loaded onto a Beckman C-18 column (5 cm x 4.6 mm id.) and eluted with 0.3%

Time	% of 0.1% TFA	% Acetonitrile
0	98	2
15	89	11
25	87	13
30	82	18
40	74	26
41	98	2

Table 7. Gradient composition used to separate the peptides generated by treatment with trypsin, clostripain, or EPLC.

TFA-acetonitrile gradient (composition in Table 8). Absorbance was monitored at 214 nm.

Amino Acid Content of the Peptides

The peptide samples were transferred to 10 ml freeze drying ampoules (Bellco) and treated with 250 µl of 6N HCl (Pierce Sequanal Grade). The solution was evacuated for 20 seconds, evacuated for 10 seconds, flushed with nitrogen gas ($N_{2(g)}$) for 4 seconds, evacuated for 10 seconds, flushed with $N_{2(g)}$ for 4 seconds, evacuated for 10 seconds and sealed under vacuum. The ampoules were baked for 1 hour at 155°C. The seal was broken and the contents freeze dried.

The hydrolyzed samples were mixed with 200 µl of deionized and distilled H_2O, 25 µl of saturated borate buffer adjusted to pH 9.5 with NaOH, 12.5 µl of O-pthaldialdehyde (OPA, composition in Table 9), and 62.5 µl of HPLC grade methanol. Within five minutes after mixing, a 35 µl aliquot was injected by a Rheodyne 7120 injector valve fitted with a 100 µl sample loop into two Waters Associate u-Bondapak C-18 columns (each was 30 cm x 3.9 mm id.) connected in series. The detectors and analysis systems were the same as those used in peptide isolation. A five step multilinear gradient consisting of acetonitrile and 0.01 M Na_2HPO_4 at pH 7.0 (see Table 10 for composition) was introduced into the system with a Varian 5060 pump.

The single letter amino acid code (Hunt et. al. 1976) will be used throughout (Table 11). The amino acid sequence of α_1-, α_2-, and β-purothionin and the expected cleavage sites from hydrolysis

Time	% CH_3CN	% 0.3% TFA
0	30	70
26	0	100

Table 8. The composition of the 0.3% TFA-acetonitrile linear gradient used to separate the peptides generated by EPLC digestion. Flow rate was 2 ml./minute.

25 mg.	o-opthaldialdehyde
4.5 ml.	HPLC grade methanol
50 ug.	ethanethiol
0.5 ml.	saturated borate buffer, pH 9.5

Table 9. Composition of orthopthaldialdehyde solution. This solution gave satisfactory results for five days if stored in a dark bottle.

Time	% CH_3CN	% 0.01 Na_2HPO_4 M
0	9	91
2	20	80
35	32	68
55	80	20
56	80	20
56.1	9	91

Table 10. Composition of the acetonitrile-phosphate buffer gradient used to determine the amino acid content of the peptides.

Amino Acid	Code Letter
Alanine	A
Glycine	G
Leucine	L
Valine	V
Proline	P
Tyrosine	Y
Methionine	M
Serine	S
Threonine	T
Phenylalanine	F
Aspartic acid	D
Glutamic acid	E
Asparagine	N
Glutamine	Q
Tryptophan	W
Pyridylethylcysteine	PC
Isoleucine	I
Lysine	K
Histidine	H
Arginine	R

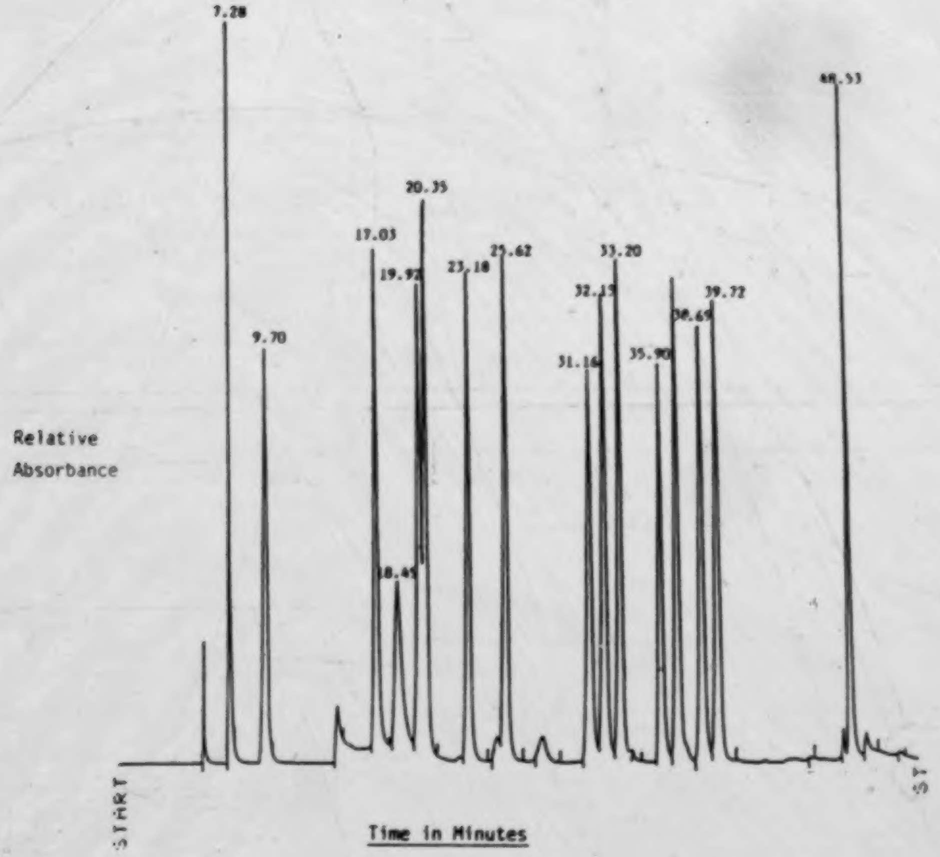
Table 11. The amino acid and code letters following Hunt et. al. (1976).

with trypsin, clostripain, and EPLC is given in Table 12.

To determine the amino acid content of each peptide, the elution profile of an amino acid calibration mixture was compared to the amino acid elution profile from each peptide. Since each amino acid elutes from the column at a characteristic position, the amino acids in a HCl treated peptide can be identified (Figure 1). The number of each amino acid residue per peptide was calculated by dividing peak area representing each amino acid by the average peak area from 6 standard mix elution profiles (Table 13). An example of the elution profile of a standard mix is given in Figure 1. All amino acids (Beckman Instrument Co.) are in equal concentrations except S-β-(4-pyridylethyl)-L-cysteine (PC), which was present at ten times the concentration of the other amino acids. The OPA derivative of PEC fluoresces about one-tenth as much as the other OPA-amino acid derivatives (Lookhart et. al. 1982). Proline does not react with OPA so its presence could not be determined.

Amino acid analysis of EPLC derived peptides of purothionin from *T. urartu* and *A. sharonensis* was similar to the procedure of Henrikson and Meradith (1984). The amino acids are derivatized with phenylisothiocyanate (PITC) and resulted in phenylthio-carbamyl (PTC) compounds (Henrikson and Meridith, 1984). Using this method, proline can be identified. Reverse phase HPLC separated all amino acids and allowed the determination of the amino acid content of each peptide.

The amino acid content of each peptide was compared with the establishment amino acid sequence of α_1-purothionin. From this, the amino acid sequence of each peptide and protein could be deduced.



Time in Minutes

Amino Acid	Time
D	7.28
E	9.70
S	17.03
H	18.49
T	19.92
G	20.35
A	23.18
Y	25.62
R	31.16
V	32.15
H	33.20
PC	35.90
I	37.04
L	38.69
F	39.72
K	48.53

Figure 1. The HPLC elution profile of a standard mix of amino acids. The amino acid and time of elution is given above.

Table 12. The amino acid sequence of α_1-, α_2-. and β-purothionin. Arrows indicate the cleavage site of trypsin ↑, EPLC ⇑, and clostripain ⇡.

* α_1- and α_2-purothionin is cut at this point by trypsin and clostripain while β-purothionin is cut at this position by trypsin and EPLC. (Jones and Mak, 1982).

Purothionin

Amino Acid Number	α_1	α_2	β	
	K	K	K	←
	S	S	S	
	C	C	C	
	C	C	C	
5	R	R	K	← ←* ↩*
	S	T	S	
	T	T	T	
	L	L	L	
	G	G	G	
10	R	R	R	← ↩
	N	N	N	
	C	C	C	
	Y	Y	Y	
	N	N	N	
15	L	L	L	
	C	C	C	
	R	R	R	← ↩
	A	S	A	
	R	R	R	← ↩
20	G	G	G	
	A	A	A	
	Q	Q	Q	
	K	K	K	← ↩
	L	L	L	
25	C	C	C	
	A	S	A	
	G	T	N	
	V	V	V	
	C	C	C	
30	R	R	R	← ↩
	C	C	C	
	K	K	K	← ↩
	I	L	L	
	S	T	T	
35	S	S	S	
	G	G	G	
	L	L	L	
	S	S	S	
	C	C	C	
40	P	P	P	
	K	K	K	← ↩
	G	G	D	
	F	F	F	
	P	P	P	
45	K	K	K	

	1	2	3	4	5	6	Mean
D	188	148	159	173	179	168	169
E	189	155	171	221	184	172	182
S	248	192	219	281	231	215	231
T	208	175	204	227	212	192	203
G	274	211	250	303	254	231	254
A	240	196	227	292	231	218	234
Y	250	207	243	301	251	232	247
R	216	202	221	286	227	207	226
V	289	223	256	299	259	241	261
I	273	230	267	319	270	251	268
L	252	212	248	309	249	231	250
F	253	212	247	301	251	232	249
K	215	185	213	271	222	202	218
PEC	20.7	17.6	20.4	23.6	20.8	19.2	20.4

Table 13. The areas of each amino acid from HPLC analysis of six standard amino acid mixtures. PEC was at ten times the concentration compared to the other amino acids and was corrected for this difference by dividing the peak areas by ten. Letters identifying each amino acid are located in the left column.

Procedure for Obtaining and Photographing Somatic Chromosomes

Seeds were induced to germinate by placing them on a ten cm base of vermiculite that had been saturated with water. The seeds were covered with about two mm of vermiculite, watered as needed, and stored at 23°C. Germination occurred in 3-6 days, depending on the species. Once the shoots emerged above the vermiculite, the seedlings were removed, the root tips cut off 4-8 mm from the tip, and placed in 4°C distilled water for 22-25 hours. The water was replaced by Carnoy's Fluid (3 parts 95% ethanol to 1 part glacial acetic acid) to fix the tissue and left at room temperature for at least 24 hours and up to one month.

The root tips were placed in a solution of 2% aceto-carmine solution (2 g carmine, 45 cm^3 glacial acetic acid, 55 cm^3 distilled water, prepared following the directions by Sharma and Sharma 1972), for at least two hours and no more than 24 hours. Root tip squashes were obtained by immersing the stained root in a drop of 45% acetic acid located on a 25 x 77 mm microscope slide (Corning Glass Inc.). The root cap was removed with a scalpel and the tissue within about 2 mm of the tip was gently macerated. During this part of the procedure, a Wild M3 dissecting microscope was used (16 power objective) and light intensity was adjusted using a Hammond microscope transformer, Model 20-D. A 22 mm square coverslip (Corning Glass Inc.) was lowered over the tissue and gently tapped with the eraser end of a pencil. The slide was scanned with a Wild M20-11612 compound photomicroscope using a 10 power objective. Light intensity was adjusted using a Hammond, Model DB6F, microscope transformer.

Photographs of appropriate somatic metaphase chromosome spreads were taken with Kodak technical pan film 2415 (Eastar-AH base) under a 40 power objective lens. Film exposure and advance was controlled by a Wild photoautomat MEL13 panel.

The length of each chromosome arm was determined using a Micro Plan II Image Analysis System (Laboratory Computer System Inc.) that had been interfaced with an IBM Personal Computer. The arm ratio was calculated and a description of the chromosomes based on the centromere location and presence or absence of a satellite which followed that by Giorgi and Bozini (1969) resulted in 4 groups:

Sat. - chromosomes with satellites (the satellite length was omitted when arm ratio was calculated).

M - medium chromosomes have an arm ratio of 1.00-1.25.

SM - submedian chromosomes have an arm ratio between 1.26-1.75.

St - subterminal chromosomes have an arm ratio greater than 1.75.

Chromosome pairs in the karyotype of each species were arranged in order of decreasing length.

RESULTS

Results of Purothionin Analysis

All seeds used in this study were from seed increased in Edmonton, Alberta between 1982 and 1986. Extraction and analysis of purothionin from *A. speltoides*, *A. longissima*, *A. bicornis*, and *A. searsii* was completed at the USDA facility at Manhattan, Kansas. The analysis of purothionin from *T. urartu* and *A. sharonensis* was completed at the USDA facility at Madison, Wisconsin.

Extraction and Analysis of Purothionin From *A. speltoides*

The results from CMC column separation of crude purothionin are given in Figure 2 and Figure 3. After freeze drying fraction 17-19 (Figure 3), 11.3 mg. of purothionin was obtained. The protein was reduced and pyridylethylated. The results of CMC column purification are given in Figure 4. After freeze drying fractions 27-30, 3.5 mg. of protein was obtained.

Trypsin Digestion

The HPLC elution pattern of reduced and pyridylethylated purothionin that had been hydrolysed with trypsin for one hour is given in Figure 5. Nine fractions were recovered. Unfortunately, the fraction containing amino acids 6-10 was not recovered. Comparing the position where amino acids 6-10 eluted after clostripain hydrolysis, this fraction probably eluted at 7.35 minutes.

Although 9 fractions were recovered, only fractions 1, 2, 3, 5, 7, and 9 were discussed. Fractions 4, 6, and 8 represented parital breakdown products from trypsin hydrolysis and were not discussed. This was the procedure followed in all the analyses.

Amino Acid Analysis

Fraction 1 contained aspartic acid, glycine, alanine, arginine, and lysine (Figure 6) and corresponded to amino acids 18-23. One lysine residue per peptide is expected yet the calculated value was 1.68. This may represent an overestimation of the peak area since another peak occurs before the lysine peak reached the baseline.

Fraciton 2 contained 1 serine, 1 arginine, 2 PEC, and 1 lysine residues per peptide (Figure 7) which corresponded to amino acids 1-5.

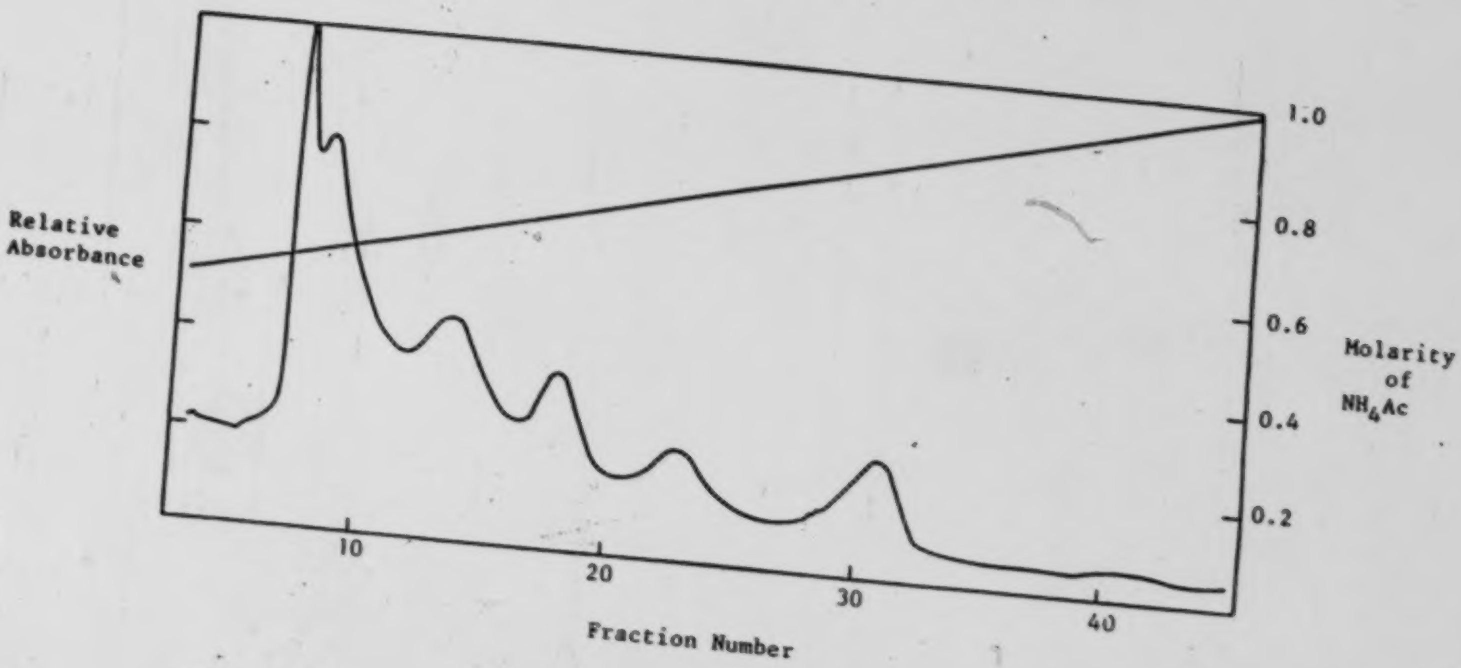


Figure 2. CMC separation of crude purothionin from A. speltoides using a 0.5-1.1 M NH_4Ac gradient, pH 5.2.

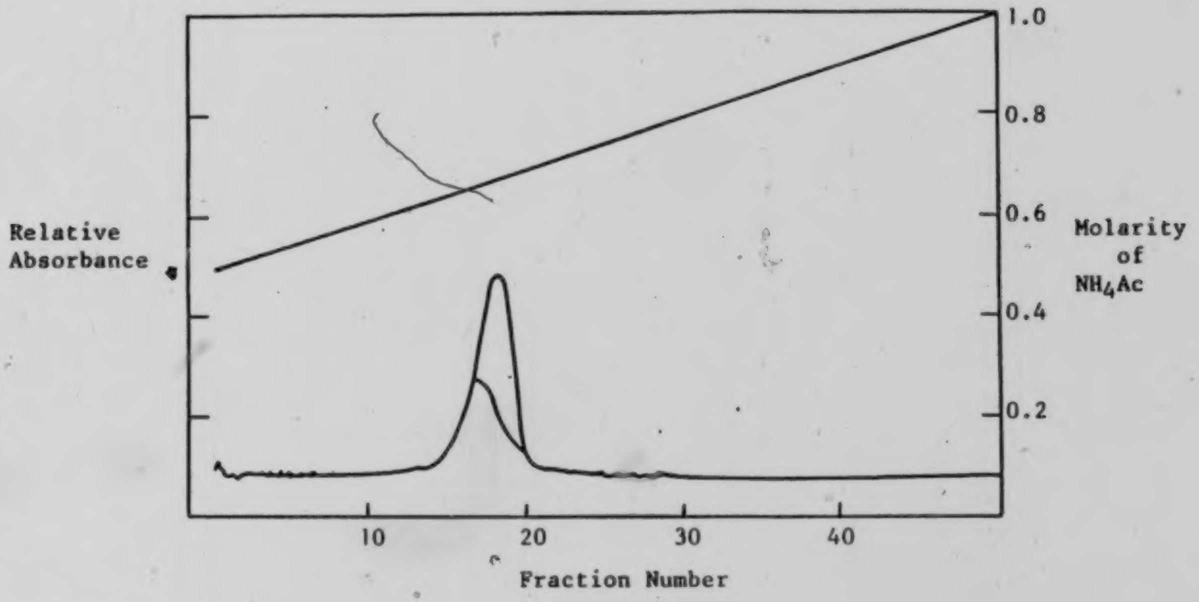


Figure 3. CMC separation of purothionin from A. speltoides using a 0.5-1.0 M NH_4Ac gradient, pH 5.2.

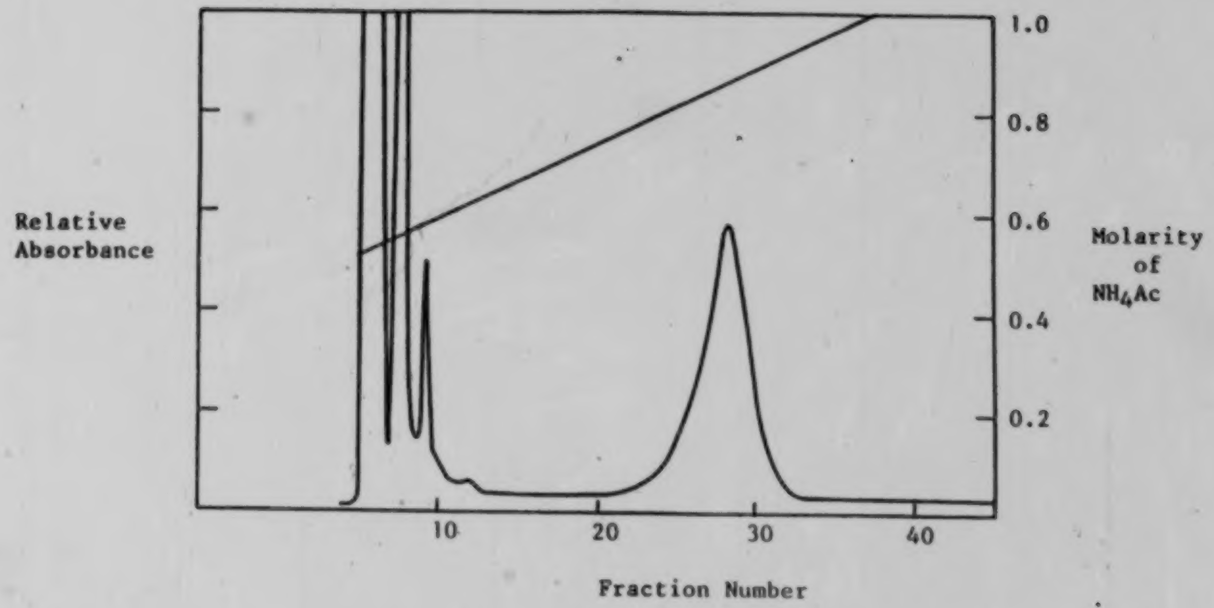


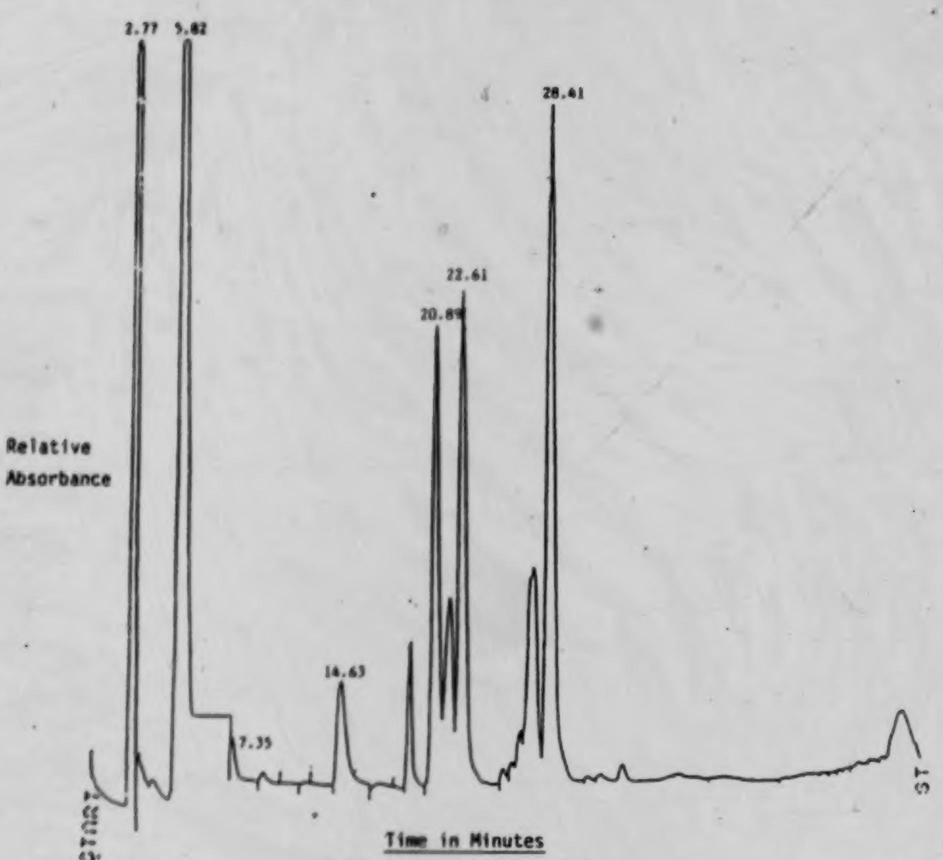
Figure 4. CMC separation of reduced and pyridylethylated purothionin from A. speltoides using a 0.5-1.1 M NH_4Ac gradient, pH 5.2.

Fraction 3 contained 1 glycine, 1 phenylalanine, and 1 lysine residue per peptide (Figure 8) and corresponded to amino acids 42-45.

Fraction 5 contained 2 serine, 1 glycine, 1 alanine, 1 valine, 1 PEC, 1 isoleucine, and 1 lysine residue per peptide (Figure 9) and corresponded to amino acids 33-41.

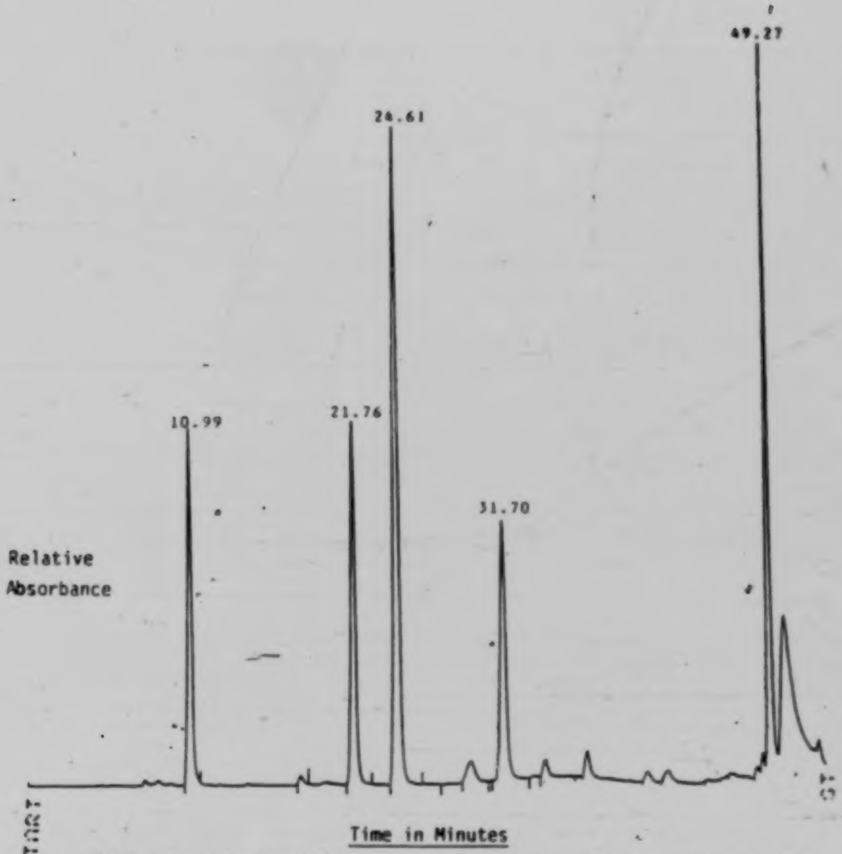
Fraction 7 contained 1 glycine, 1 alanine, 1 arginine, 1 valine, 2 PEC and 1 leucine residue per peptide (Figure 10) and corresponded to amino acids 24-30. Although the calculated value for PEC was 2.51, it was felt that 2 residues per peptide existed. When fraction 8 from clostripain treatment was analyzed, 2 PEC residues per peptide were found in the peptide containing amino acids 20-30.

Fraction 9 contained 2 aspartic acid, 1 tyrosine, 1 arginine, 2 PEC, and 1 leucine residue per peptide (Figure 11) and corresponded to amino acids 11-17. Although the calculated value for PEC was 1.48, analysis of amino acids 11-17 after clostripain hydrolysis (Fragment 8) reveals that 2 residues existed.



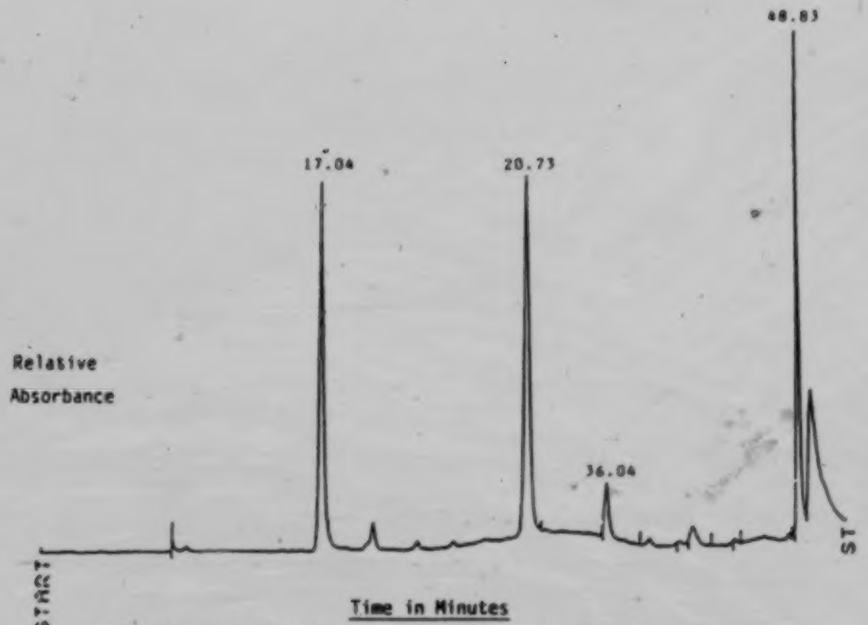
Fraction	Time (min.)
2	5.82
3	14.63
5	20.89
7	22.61
9	28.41

Figure 5. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. speltoides* that had been treated with trypsin for 1 hour.



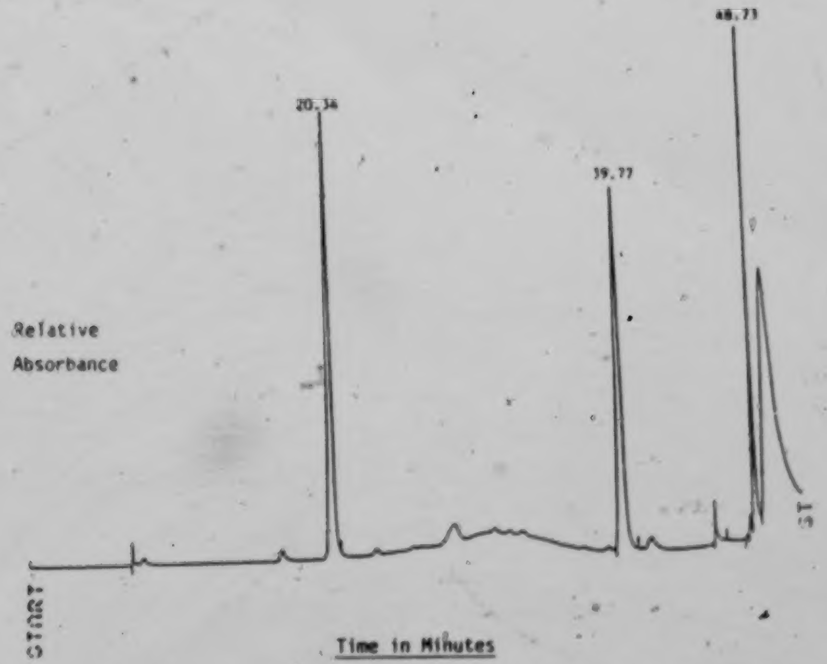
Amino Acid	Time (min.)	Area	Area/Standard	Ifl=8.18	Whole Number
E	10.99	1723	9.47	1.16	1
G	21.76	1998	7.87	0.96	1
A	24.61	3866	16.52	2.02	2
R	31.70	1918	8.49	1.04	1
K	49.27	2989	13.71	1.68	2

Figure 6. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 1 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



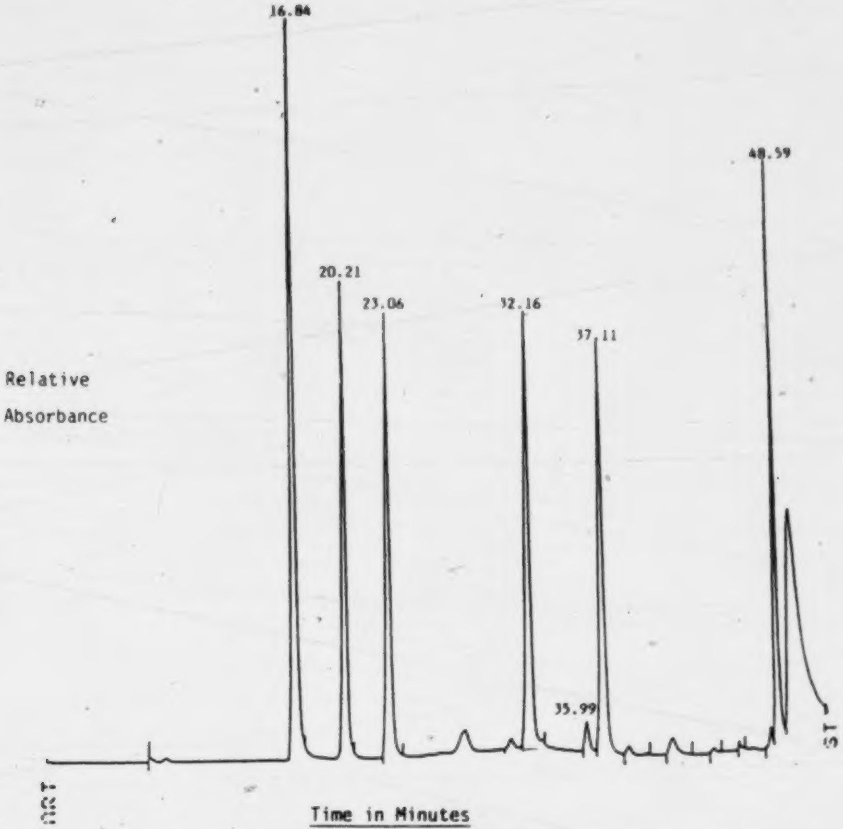
Amino Acid	Time (min.)	Area	Area/Standard	If1=3.85	Whole number
S	17.04	797	3.45	0.89	1
R	30.73	1144	5.08	1.32	1
PC	36.04	129	6.32	1.64	2
K	48.83	925	4.24	1.10	1

Figure 7. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 2 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



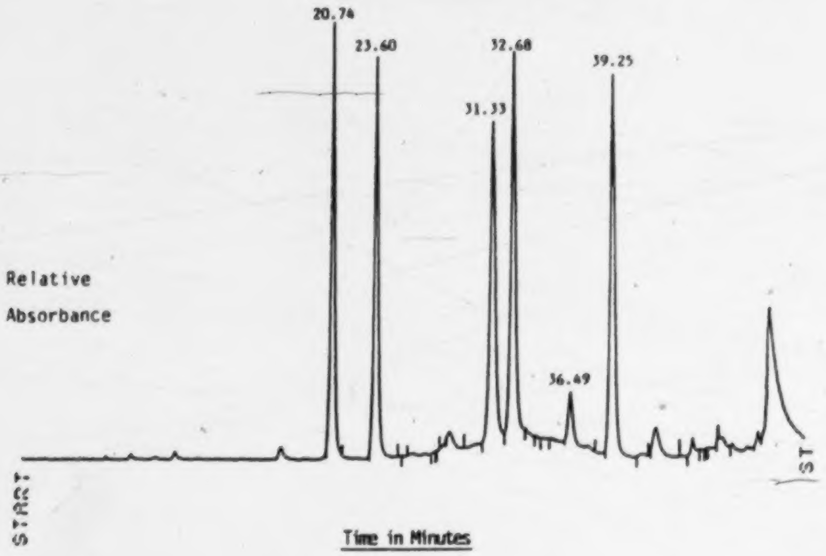
Amino Acid	Time (min.)	Area	Area/Standard	[f]=7.85	Whole Number
G	20.34	1962	7.72	0.98	1
F	39.77	2079	8.35	1.06	1
K	48.73	1740	7.98	1.02	1

Figure 8. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



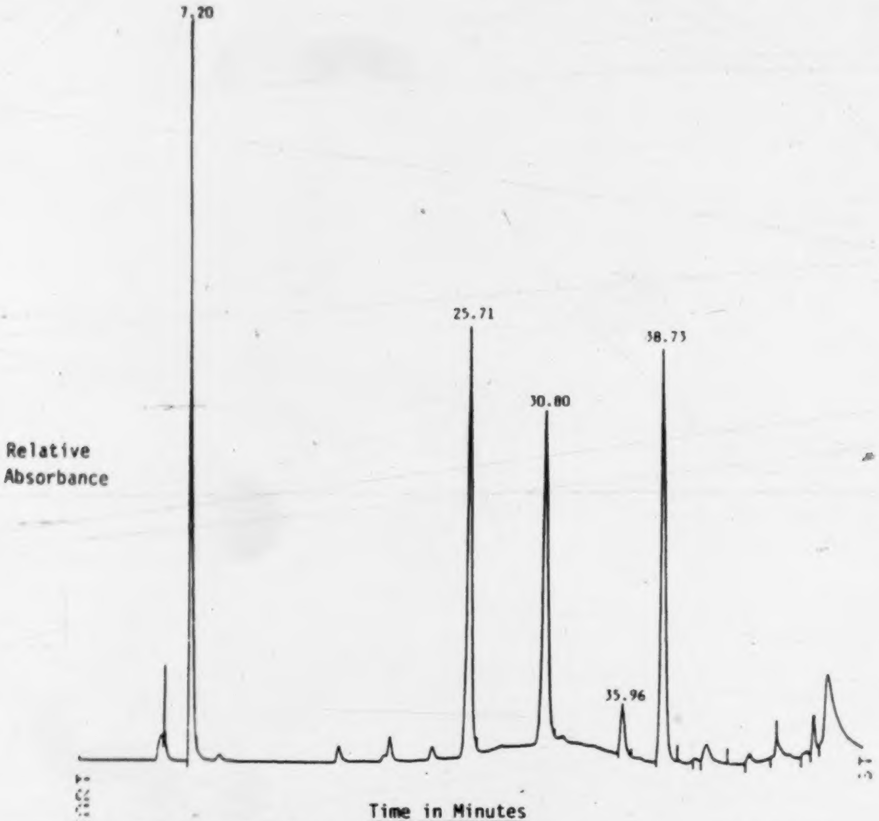
Amino Acid	Time (min.)	Area	Area/Standard	Ifl=8.19	Whole Number
S	16.84	3670	15.89	1.94	2
G	20.21	2115	8.33	1.02	1
A	23.06	2275	9.72	1.19	1
V	32.16	2537	9.72	1.19	1
PC	35.99	164	8.04	0.98	1
I	37.11	2493	9.30	1.14	1
K	48.59	2038	9.35	1.14	1

Figure 9. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=4.14	Whole Numbers
G	20.74	1005	3.96	0.96	1
A	23.60	1036	4.43	1.07	1
R	31.33	1057	4.68	1.13	1
V	32.68	1197	4.59	1.10	1
PC	36.49	212	10.39	2.51	2
L	39.25	1081	4.32	1.04	1

Figure 10. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 7 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	Ifl=8.21	Whole Number
D	7.20	3038	17.98	2.19	2
Y	25.71	2036	8.24	1.00	1
R	30.80	1848	8.18	1.00	1
PC	35.96	247	12.11	1.48	2
L	38.73	2271	9.08	1.11	1

Figure 11. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 9 that resulted from trypsin digestion of pyridylethylated pyrothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.

Clostripain Hydrolysis

The HPLC elution pattern of reduced and pyridylethylated purothionin that had been treated with clostripain for 30 minutes is given in Figure 12. Twelve fractions were recovered.

Amino Acid Analysis

Fraction 1 contained 1 alanine and 1 arginine residue per peptide (Figure 13) and corresponded to amino acids 18 and 19. The peak that emerged at 46.90 minutes was an artifact.

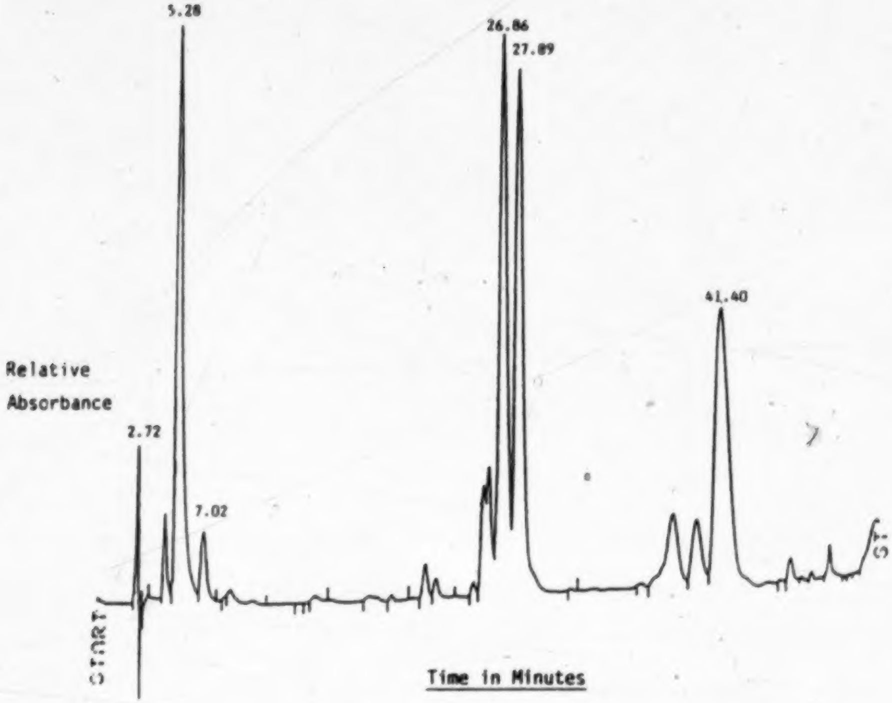
Fraction 3 contained 1 serine, 1 arginine, and 1 lysine residue per peptide (Figure 14) and corresponded to amino acids 1-5. The peak at 36.72 minutes representec PC but the area calculated appears to be inaccurate. However, fraction 2 from trypsin hydrolysis indicated that two PE residues existed in this peptide.

Fraction 4 contained serine, threonine, glycine, arginine, and leucine in equal concentrations. The calculated value for glycine was 1.81 residues per peptide. This was an overestimation since threonine and glycine did not elute separately and some of the threonine peaks are included in the glycine peak area (Figure 15).

Fraction 8 contained 2 residues per peptide of glycine, alanine, and PEC, and 1 residue per peptide of glutamic acid, arginine, valine, leucine and lysine (Figure 16) which corresponded to amino acids 20-30.

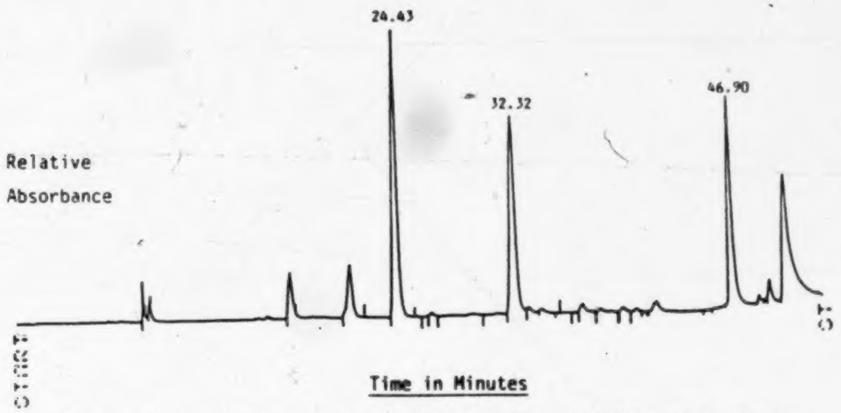
Fraction 9 contained 2 aspartic acid, 1 tryosine, 1 arginine, 2 PEC, and 1 leucine residue per peptide (Figure 17) and corresponded to amino acids 11-17. The chart paper on the printer plotter which monitored at 220 nm. was caught in the system and consequently the peak for aspartic acid was not printed. Nevertheless, the area was calculated and the amino acid value was obtained. The chart from the printer plotter which measured relative fluorescence at 254 nm is given in figure 17.

Fraction 12 contained 3 lysine, 2 serine, 2 glycine, and 2 PC residues per peptide and 1 valine, 1 isoleucine, 1 alanine and 1 phenylalanine residue per peptide (Figure 18) which corresponded to amino acids 31-45.



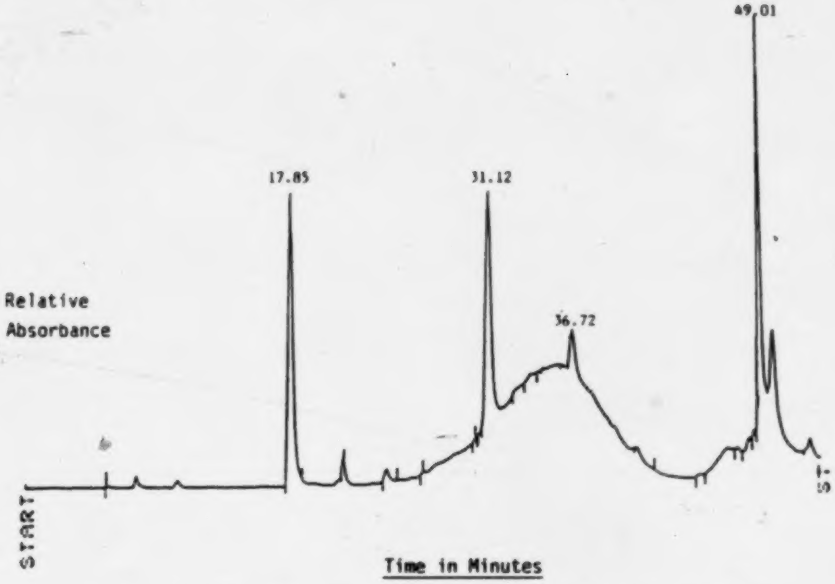
Fraction	Time
1	2.72
3	5.28
4	7.02
8	26.86
9	27.89
12	41.40

Figure 12. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. speltoides* that had been treated with clostripain for 30 minutes. Fraction number and elution time is given above.



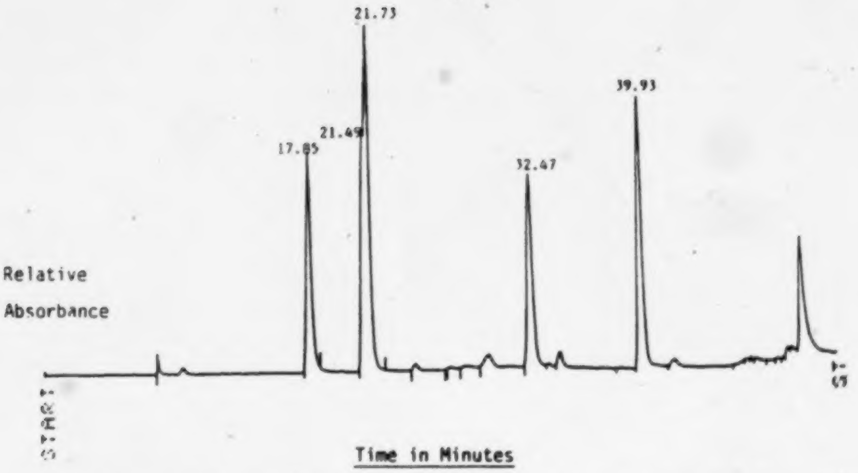
Amino Acid	Time (min.)	Area	Area/Standard	If1=6.01	Whole Number
A	24.43	1586	6.78	1.13	1
R	32.32	1358	6.01	1.00	1

Figure 13. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 1 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



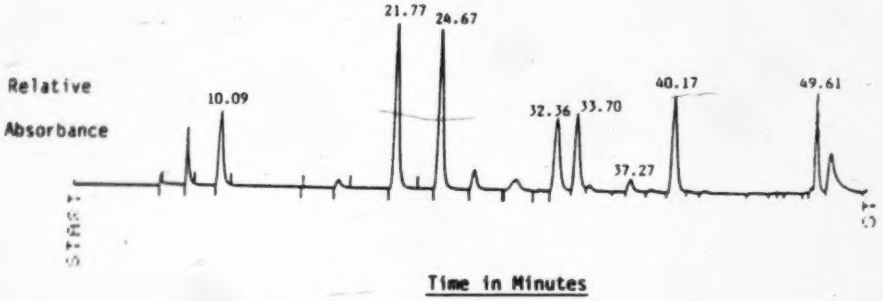
Amino Acid	Time (min.)	Area	Area/Standard	If1=7.24	Whole Number
S	17.85	1672	7.24	1.00	
R	31.12	1882	8.33	1.15	
PEC		361	17.69	2.44	
K	49.01	2218	10.17	1.41	

Figure 14. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



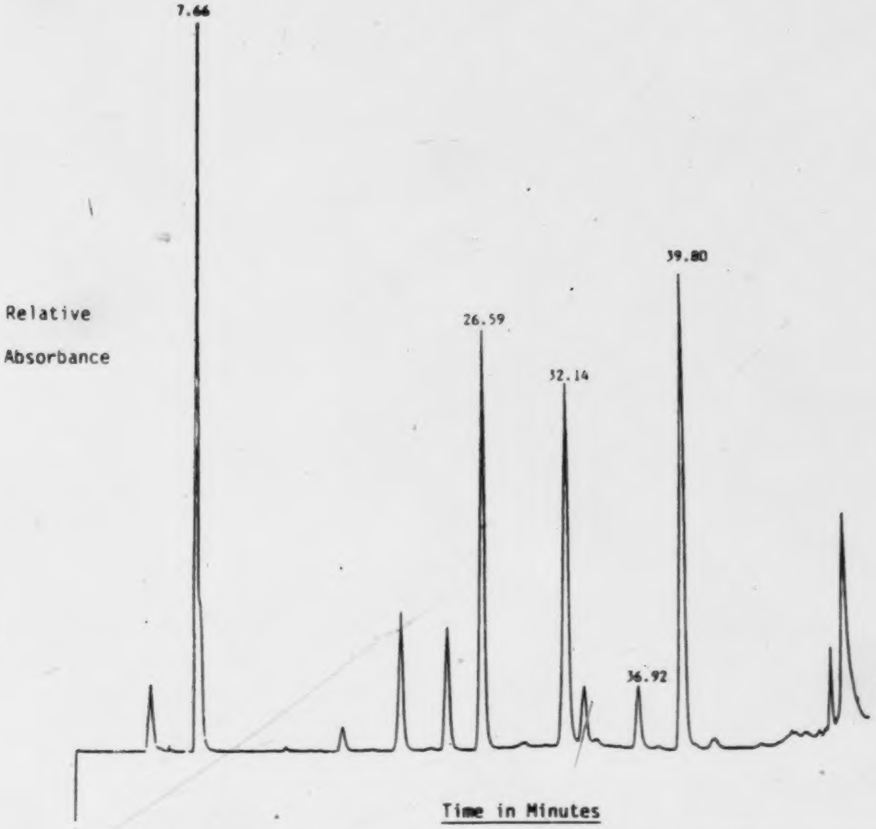
Amino Acid	Time (min.)	Area	Area/Standard	If1=4.58	Whole Number
S	17.85	1254	5.43	1.19	1
T	21.49	758	3.73	2.81	1
G	21.73	2116	8.33	1.81	2
R	32.47	1357	6.00	1.31	1
L	39.93	1683	6.73	1.47	1

Figure 15. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 4 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



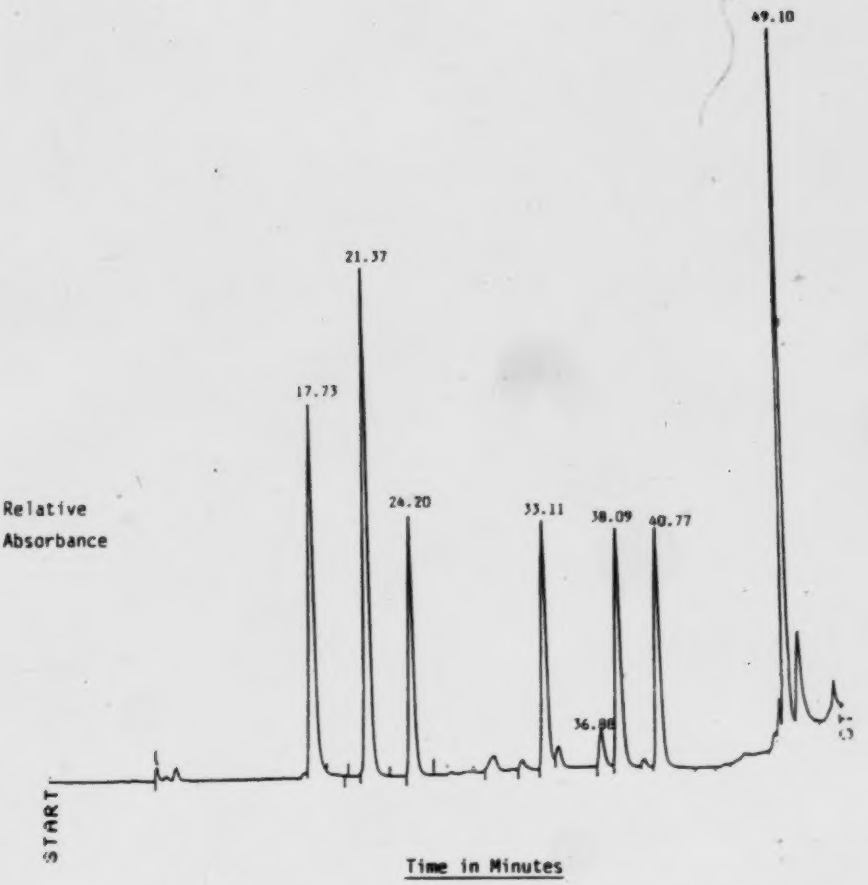
Amino Acid	Time(min.)	Area	Area/Standard	Ifl=7.00	Whole Number
E	10.09	1521	8.36	1.19	1
G	21.77	3507	13.81	1.97	2
A	24.67	3541	15.13	2.16	2
R	32.36	2006	8.88	1.27	1
V	33.70	1897	7.27	1.04	1
PEC	37.27	335	16.42	2.35	2
L	40.17	2327	9.31	1.33	1
K	49.61	14.68	6.73	0.96	1

Figure 16. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 8 that resulted from clostripain digestion of pyridylethylated purothionin extracted from*A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	Ifl=7178	Whole Number
D	7.66	3225	19.08	2.45	2
Y	26.59	1793	7.26	0.93	1
R	32.59	1875	8.29	1.07	1
PEC	36.92	308	15.09	1.94	2
L	39.80	2182	8.51	1.09	1

Figure 17. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 9 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	Ifl=6.05	Whole Number
S	17.73	23.7	10.03	1.66	2
G	21.37	2843	11.19	1.85	2
A	24.20	1468	6.27	1.04	1
V	33.11	1608	6.16	1.02	1
PEC	36.88	306	15.00	2.48	2
I	38.09	1622	6.05	1.00	1
F	40.77	1544	6.20	1.02	1
K	49.10	3743	17.17	2.84	3

Figure 18. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 12 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.

EPLC Treatment

The HPLC elution pattern of reduced and pyridyethylated purothion from A. speltoides treated with EPLC for 4 hours is given in figure 19. Twelve fractions were included and represented amino acids 2-32 and 42-45. By comparing this elution pattern with that of EPLC treated A. searsii, the fraction containing amino acids 33 to 41 likely eluted at 25 min.

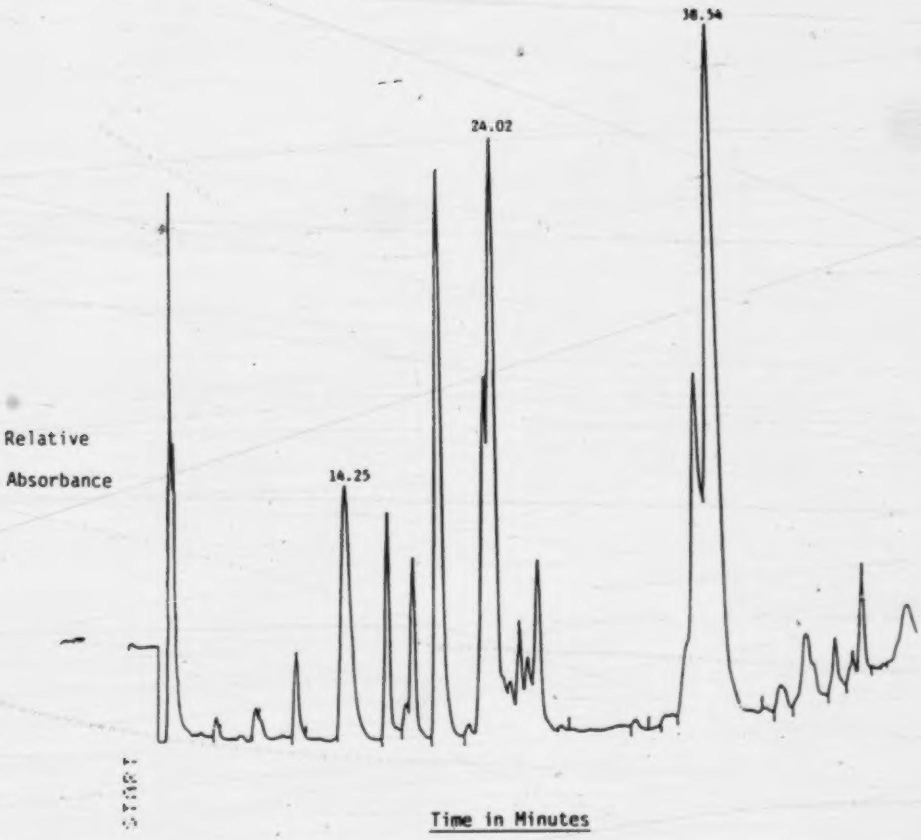
Amino Acid Analysis

Fraction 3 contained 1 glycine, 1 phenylalanine, and 1 lysine residue per peptide (Figure 20) and represented amino acids 42-45.

Fraction 8 contained 3 PEC, 1 glycine, 1 alanine, 1 arginine, 1 valine, 1 leucine, and 1 lysine residue per peptide (Figure 21) and represented amino acids 24-32.

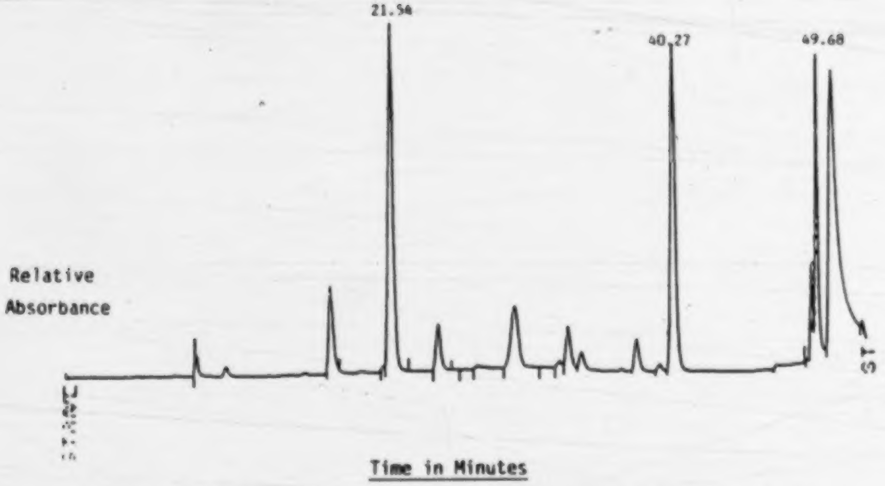
Fraction 11 contained 4 arginine, 4 PEC, 2 aspartic acid, 2 serine, 2 glycine, 2 alanine, and 2 leucine residue per peptide and 1 glutamic acid, 1 tyrosine, and 1 lysine residue per peptide (Figure 22). Glutamic acid and aspartic acid came off together in one peak. The standard area for this peak was calculated by adding 2 x aspartic acid and 1 glutamic acid standard and dividing by three. Analysis of fraction 1 and 9 from trypsin digestion and fractions 1, 8, and 9 from clostripain treatment indicated that 2 aspartic acid and 1 glutamic acid residues occur in this sequence. Calculations indicate that 5 arginine and 5 PEC residues occur in this sequence. Based on the analysis of fractions 1, 3, 4 and 9 from the clostripain digestion and fractions 1, 2 and 9 from the trypsin treatment, there are only 4 residues of each in this peptide.

The amino acid sequence of purothionin from _A_. _speltoides_ is given in Figure 23. It differs from α_1 purothionin by a leucine to valine substitution at positon 37 and a serine to alanine substitution at positon 34, 35, or 38.



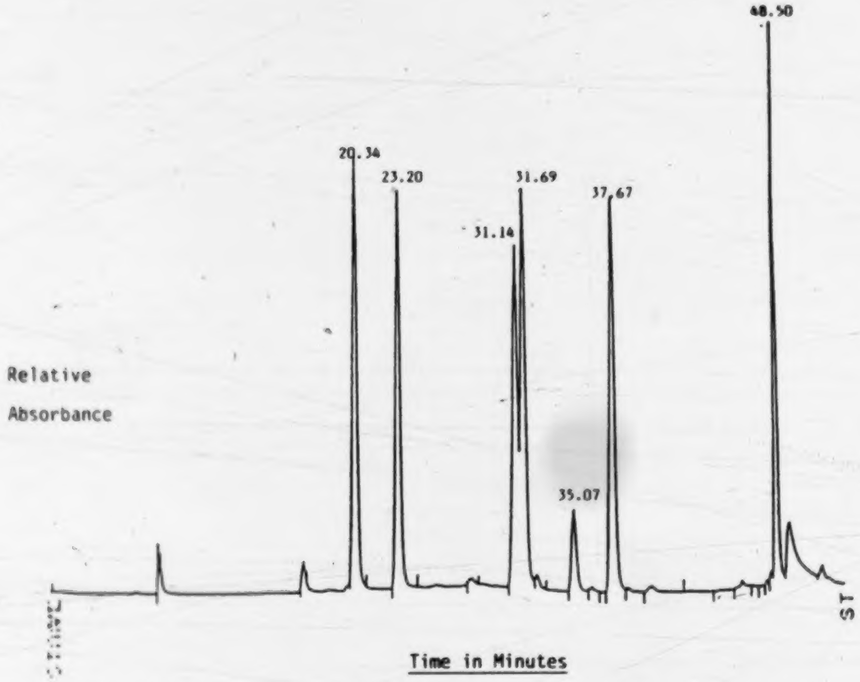
Fraction	Time
3	14.25
8	24.02
11	38.54

Figure 19. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. speltoides* that had been treated with EPLC for 4 hours. Fraction number and elution time is given above.



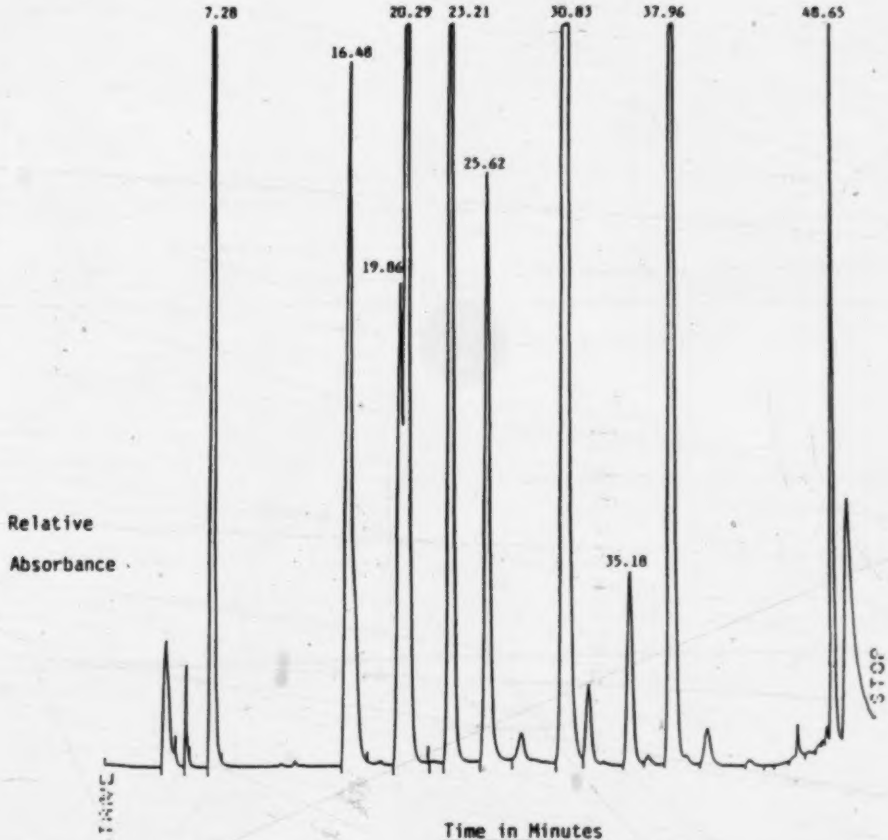
Amino Acid	Time (min.)	Area	Area/Standard	Ifl=6.54	Whole Number
G	21.54	2073	8.16	1.25	1
F	40.27	1996	8.02	1.23	1
K	49.68	1103	5.06	0.77	1

Figure 20. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=9.08	Whole Number
G	20.34	2280	8.98	0.99	1
A	23.20	2287	9.77	1.08	1
R	31.14	2072	9.17	1.01	1
V	31.69	2543	9.74	1.07	1
PEC	35.07	495	24.27	2.67	3
L	37.67	2466	9.86	1.09	1
K	48.50	2094	9.61	1.06	1

Figure 21. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 8 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	Ifl=11.86	Whole Number
D + E	7.28	7056	40.79	3.44	3
S	16.48	5236	22.67	1.91	2
T	19.86	2368	11.67	2.98	1
G	20.29	7056	27.70	2.34	2
A	23.21	6394	27.33	2.30	2
Y	25.62	3621	14.66	1.24	1
R	30.83	13500	59.74	5.03	5
PEC	35.18	1293	63.38	5.34	5
L	37.96	7316	29.26	2.47	2
K	48.65	2627	12.05	1.02	1

Figure 22. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 11 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. speltoides*. Calculations of the number of amino acid residues per peptide is given above.

K
S
C
C
5 R
S
T
L
G
10 R
N
C
Y
N
15 L
C
R
A
R
20 G
A
Q
K
L
25 C
A
G
V
C
30 R
C
K
I
A *
35 S *
G
V
S *
C
40 P
K
G
F
P
45 K

Figure 23. The amino acid sequence of purothionin from *A. speltoides*. *The serine to alanine substitution could occur at position 34, 35, or 38.

Extraction and analysis of purothionin from *A. longissima*

The results from CMC column separation of crude purothionin are given in Figure 24 and Figure 25. After freeze drying fractions 18 and 19, 3.3 mg of purothionin was obtained. The protein was reduced and pyridylethylated and 1.3 mg of purothionin was obtained after freeze drying fractions 20-26 (Figure 26).

Trypsin Digestion

The HPLC elution pattern of reduced and pyridylethylated purothionin that had been hydrolysed with trypsin for 1 hour is given in Figure 27. Seven fractions were collected and these represented all the amino acids contained in the purothionin except residues 6-10. By comparing this elution profile to that from A. speltoides purothionin digested with trypsin (Figure 5), the fraction containing amino acids 6 to 10 eluted at 7.23 or 9.13 minutes.

Amino acid content of each peptide

Fraction 1 contained glutamic acid, serine, glycine, alanine, arginine, and lysine residues (Figure 28) and corresponded to amino acids 18-23. Unfortunately, the relative amounts could not be accurately calculated. However, analysis of fraction 9 from EPLC treatment indicates 1 residue per peptide of each amino acid.

Fraction 2 contains 1 serine, 1 arginine, 2 PC, and 1 lysine residue per peptide (Figure 29) and corresponded to amino acids 42-45.

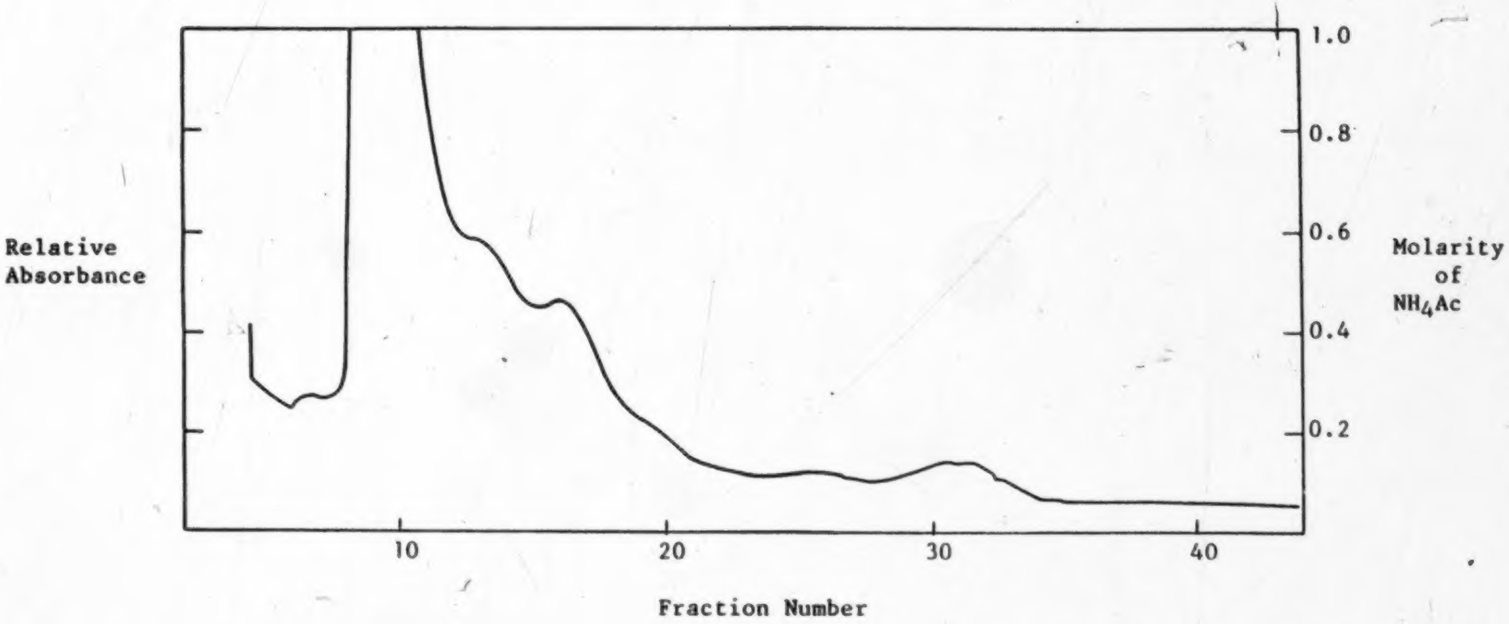


Figure 24. CMC separation of crude purothionin from A. longissima using a 0.5-1.1 M NH_4Ac gradient, pH 5.2.

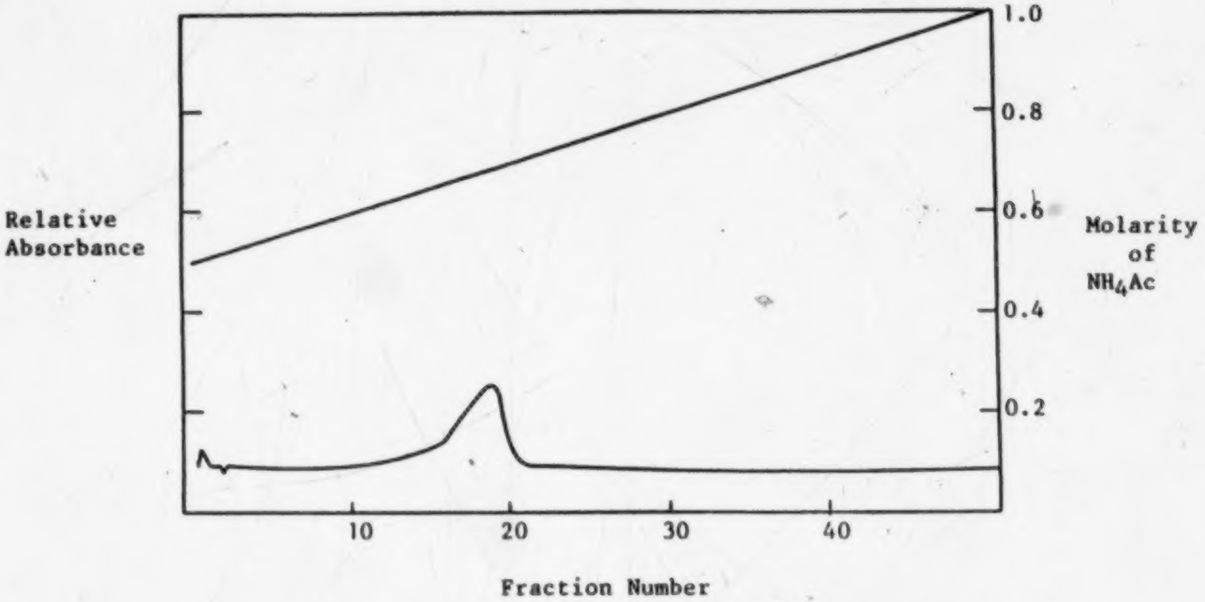


Figure 25. CMC separation of purothionin from A. longissima using a 0.5-1.1M NH_4Ac gradient, pH 5.2.

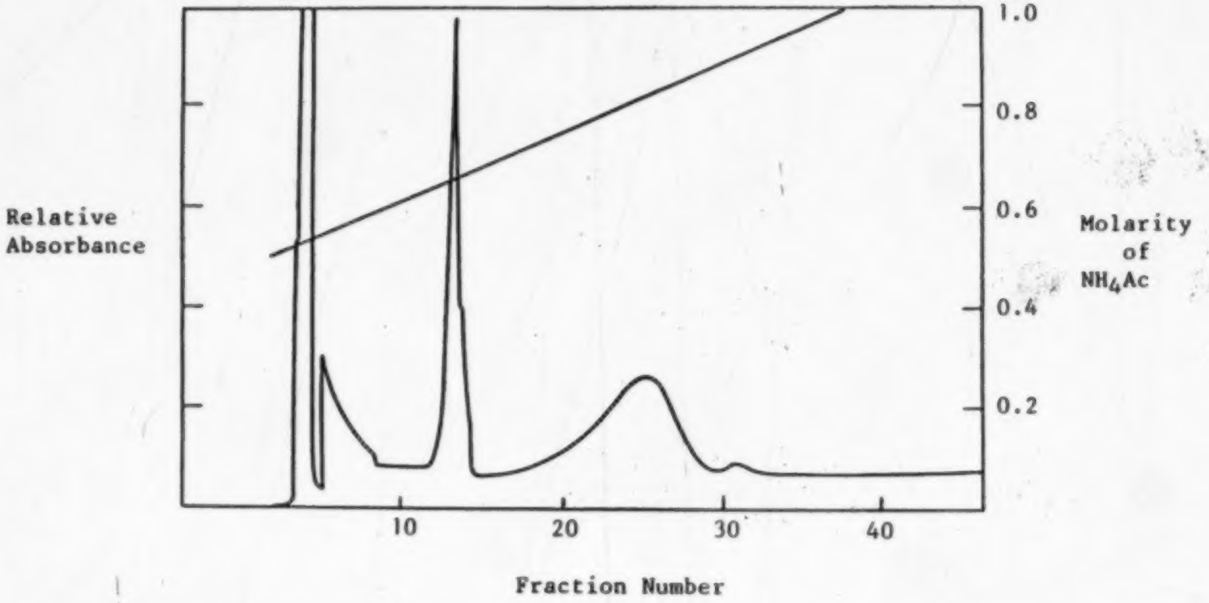
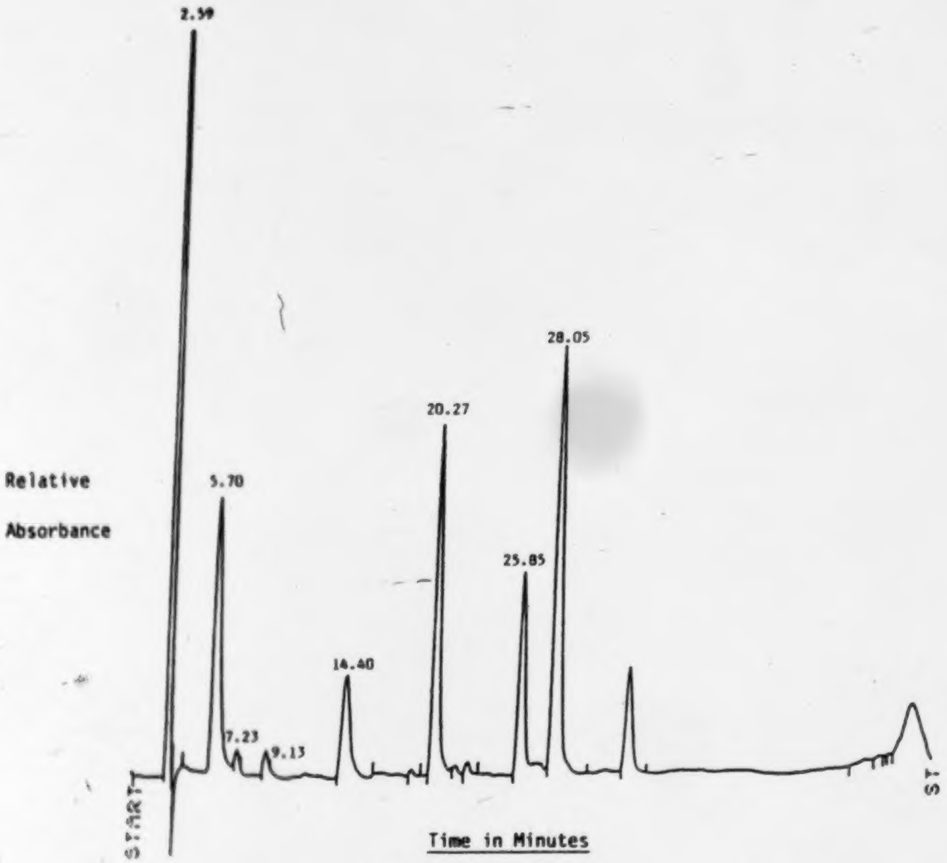
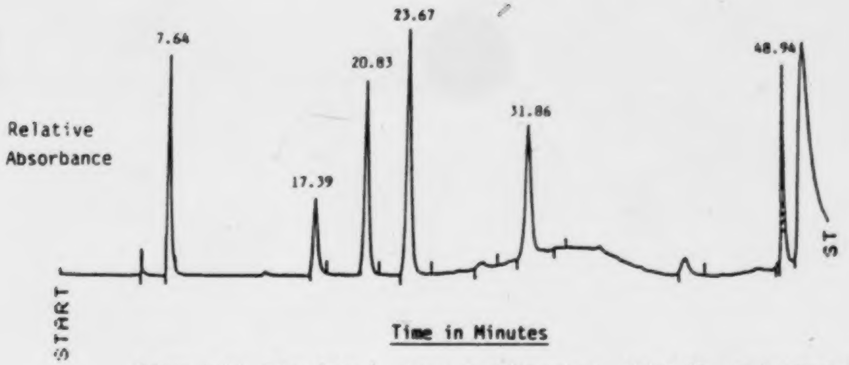


Figure 26. CMC separation of reduced and pyridylethylated purothionin from A. longissima using a 0.5-1.1M NH_4Ac gradient, pH 5.2.



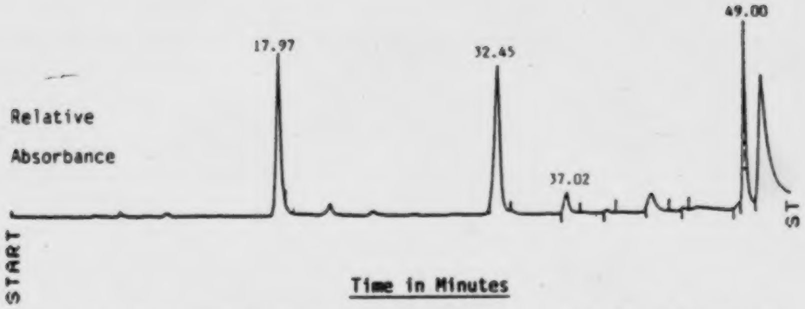
Fraction	Minutes
1	2.59
2	5.70
3	14.40
4	20.27
5	25.85
6	28.05

Figure 27. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. longissima* that had been treated witn trypsin for 1 hour.



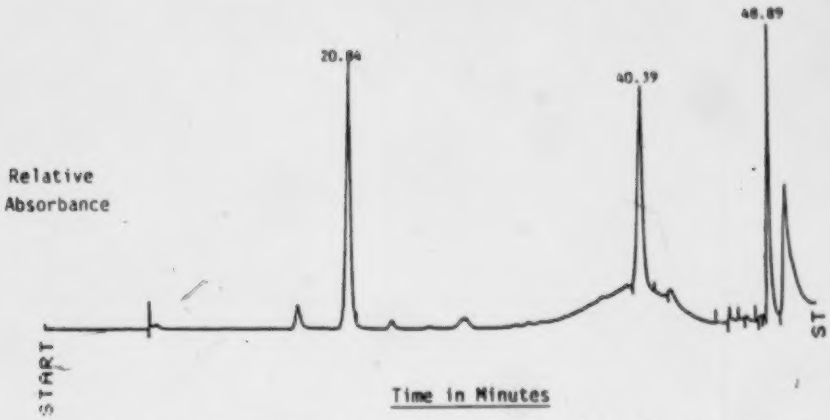
Amino Acid	Time (min.)	Area	Area/Standard	Ifl=2.97	Whole Number
E	7.64	841	4.62	1.56	1
S	17.39	409	1.77	0.59	1
G	20.83	976	3.84	1.29	1
A	23.67	1443	6.17	2.08	2
R	31.86	1010	4.47	1.51	1
K	48.94	732	3.36	1.31	1

Figure 28. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 1 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (Min.)	Area	Area/Standard	If1=3.24	Whole Number
S	17.97	755	3.27	1.01	1
R	32.45	939	4.16	1.28	1
PC	37.02	119	5.83	1.79	2
K	49.00	700	3.21	0.99	1

Figure 29. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 2 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=4.89	Whole Number
G	20.84	1198	4.72	0.97	1
F	40.39	1332	5.35	1.09	1
K	48.89	1103	5.05	1.03	1

Figure 30. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.

Fraction 3 contained 1 glycine, 1 phenylalanine, and 1 lysine residue per peptide (Figure 30) and corresponded to amino acids 42-45.

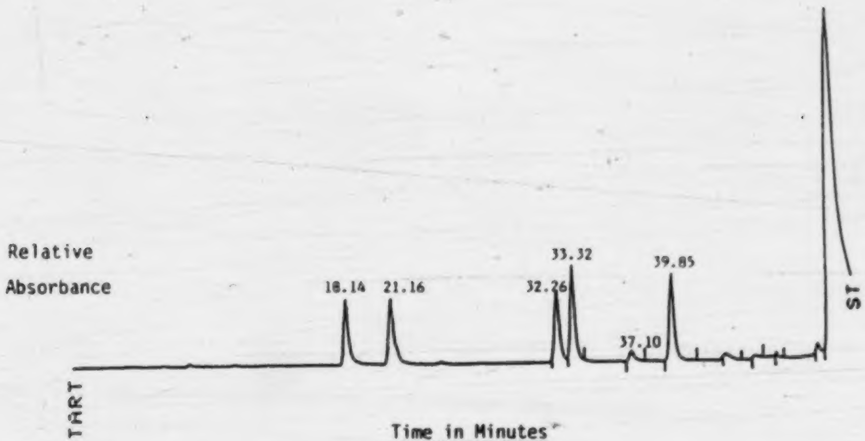
Fraction 4 contained 1 serine, 1 threonine, 1 arginine, 1 valine, 2 PC, and 1 leucine residue per peptide (Figure 31) and corresponded to amino acids 24-30. The areas for serine and threonine were underestimated and the relative amounts could not be calculated. However, when fraction 5 from clostripain hydrolysis (Figure includes amino acids 20-30), or fraction 5 from EPLC hydrolysis (Figure 41, includes amino acids 24-32) is analysed, 1 serine and 1 threonine residue per peptide exists.

Fraction 5 contained 2 serine, 1 threonine, 1 glycine, 1 PC, 2 leucine, and 1 lysine residue per peptide (Figure 32) and corresponded to amino acids 32-41.

Fraction 6 contained 2 aspartic acid, 1 tyrosine, 1 arginine, 2 PC, and 1 leucine residue per peptide (Figure 33) and corresponded to amino acids 11-17. Although the calculated value for PC was 1.25 residues per peptide, this value may be an underestimation since analysis of fraction 6 from clostripain hydrolysis indicated that two PC residues occur in this peptide. In addition, the rise in the baseline during the time when PC eluted from the column may have been led to an incorrect area calculation.

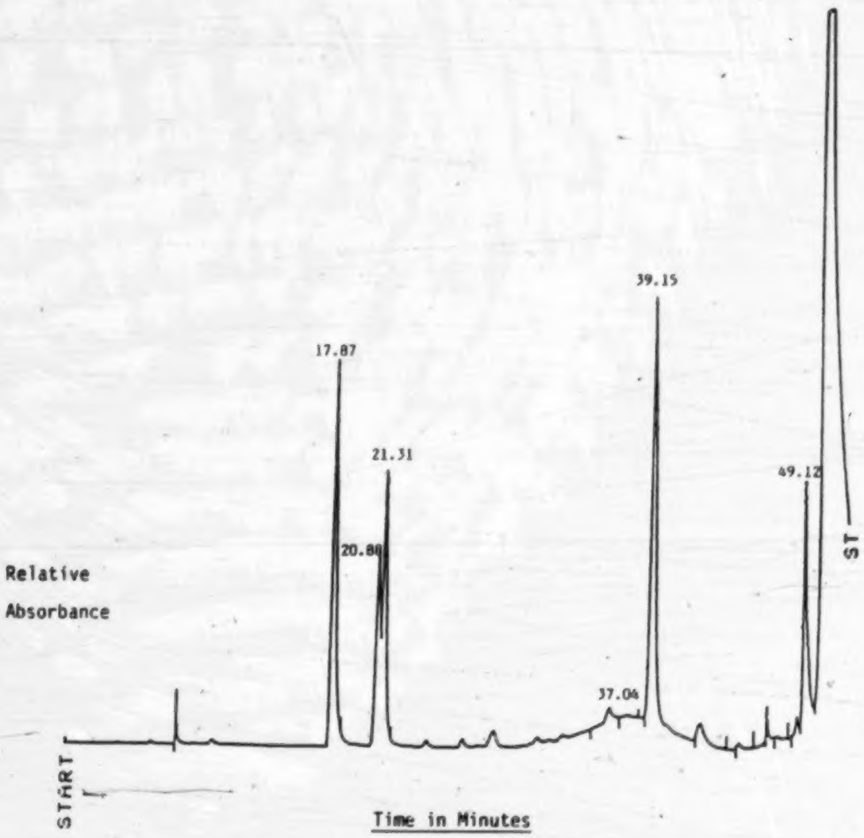
Clostripain Hydrolysis

The HPLC elution profile of clostripain treated purothionin is given in Figure 34. Ten fractions were recovered. Four of these fractions contained all amino acids present in the purothionin except amino acids 6-10. If a comparison is made with clostripain



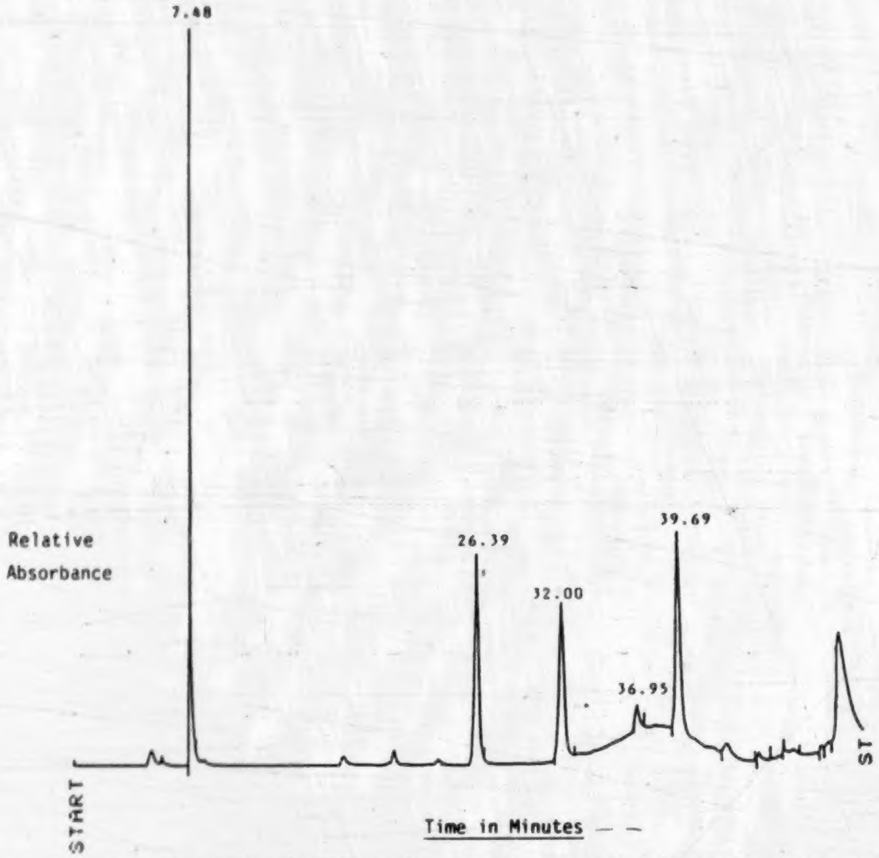
Amino Acid	Time (min.)	Area	Area/Standard	If1=1.98	Whole Number
S	18.14	80	0.01		
T	21.16	129	0.64		
R	32.26	404	1.79	0.90	1
V	33.32	531	2.03	1.03	1
PC	37.10	63	3.09	1.56	2
L	39.85	529	2.11	1.07	1

Figure 31. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 4 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=4.59	Whole Number
S	17.87	1870	8.09	1.76	2
T	20.88	834	4.11	0.89	1
G	21.31	1280	5.03	1.09	1
PC	37.04	84	4.16	0.91	1
L	39.15	2575	10.30	2.24	2
K	49.12	1011	4.64	1.01	1

Figure 32. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from trypsin digestion of pyridylethylated pyrothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=3.87	Whole Number
D	7.48	1365	8.08	2.08	2
Y	26.39	955	3.87	1.00	1
R	32.00	969	4.29	1.11	1
PC	36.95	99	4.85	1.25	1
L	39.69	1144	4.58	1.18	1

Figure 33. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.

hydrolysis of A. speltoides purothionin, the fraction that contained this peptide may occur at the end of the 5.10 minute peak, or in the 6.92 or 8.84 minute peaks.

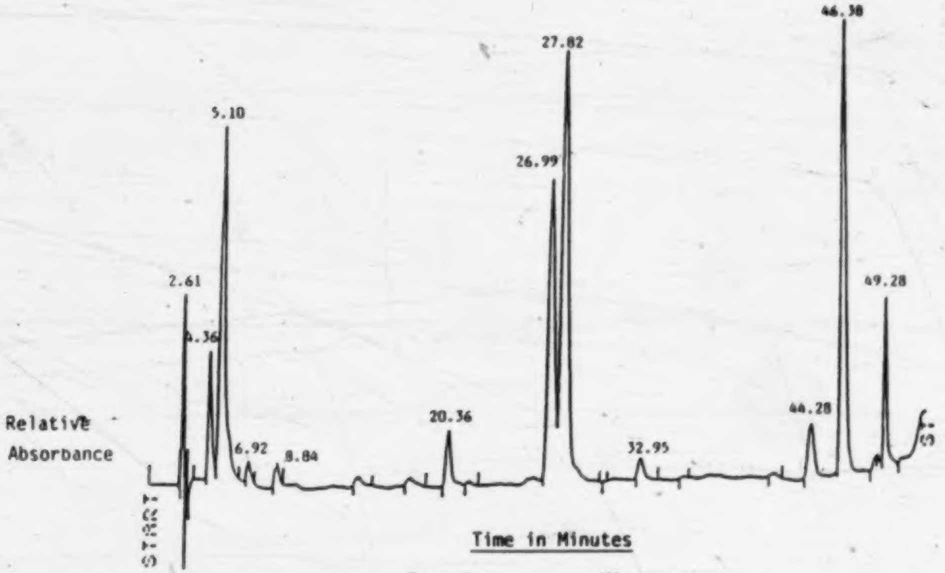
Amino Acid Content of Each Peptide

Fraction 3 contained 1 lysine, 1 serine, 2 PC, and 1 arginine residue per peptide (Figure 35) and corresponded to amino acids 1-5.

Fraction 5 contained 1 residue each of glycine, glutamic acid, serine, alanine, arginine, valine, leucine and lysine (Figure 36) and corresponded to amino acids 20-30. Threonine and glycine did not separate completely so the areas calculated are an underestimate for threonine and an overestimate for glycine.

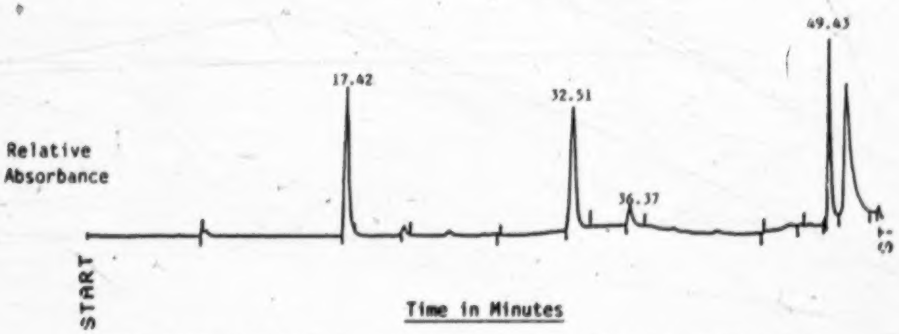
Fraction 6 contained 2 aspartate, 2 PC, 1 tyrosine, 1 arginine, and 1 leucine residue per peptide (Figure 37) and corresponded to amino acids 11-17.

Fraction 9 contained 2 serine, 2 glucine, 1 threonine, 2 PC, 2 leucine, 1 phenylalaine, and 2 lysine residues per peptide (Figure 38) and corresponded to amino acids 31-45. Threonine and glycine did not separate completely but analysis of fraction 6 and 9 from EPLC hydrolysis indicated 2 glycine and 1 serine residue per peptide (Figure 42). The calculated value for lysine is 2.12 residues per peptide. Analysis of fractions 5, 6, and 2 from EPLC hydrolysis indicated the existence of 3 lysine residues in the peptide that included amino acids 24-45. The terminal lysine may have been cleaved with clostripain and fraction 9 may represent amino acids 31-44.



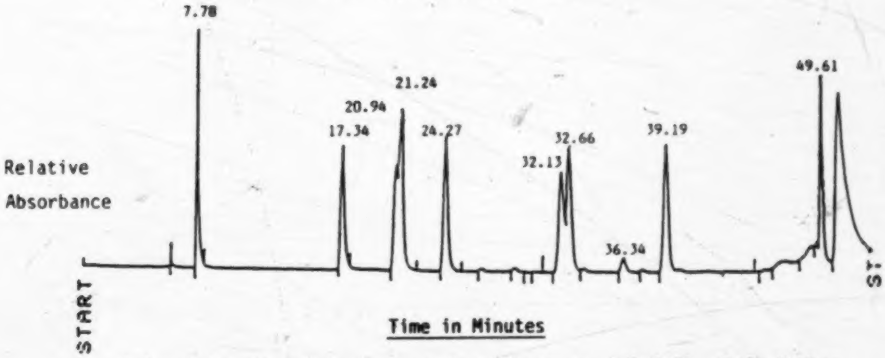
Fraction	Minutes
3	5.10
5	26.99
6	27.82
9	46.38

Figure 34. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. longissima* that had been treated with clostripain for 30 minutes.



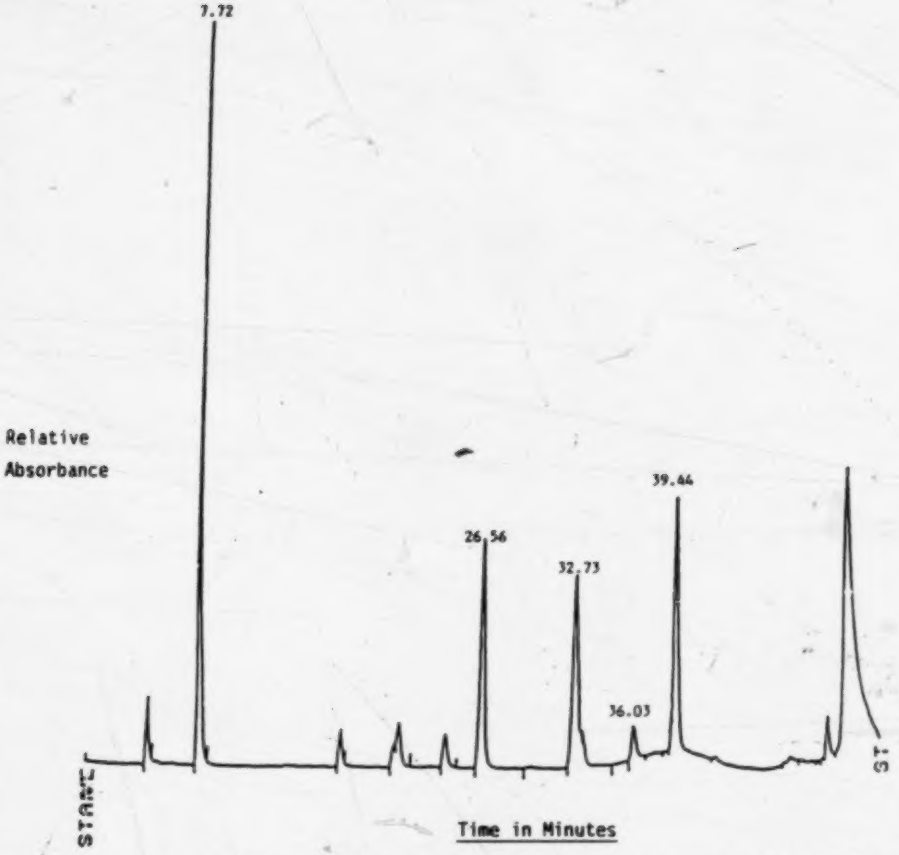
Amino Acid	Time (min.)	Area	Area/Standard	If1=3.19	Whole Number
S	17.42	739	3.19	1.00	1
R	32.51	770	3.41	1.07	1
C	36.37	101	4.95	1.55	2
K	49.43	694	3.18	0.99	1

Figure 35. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



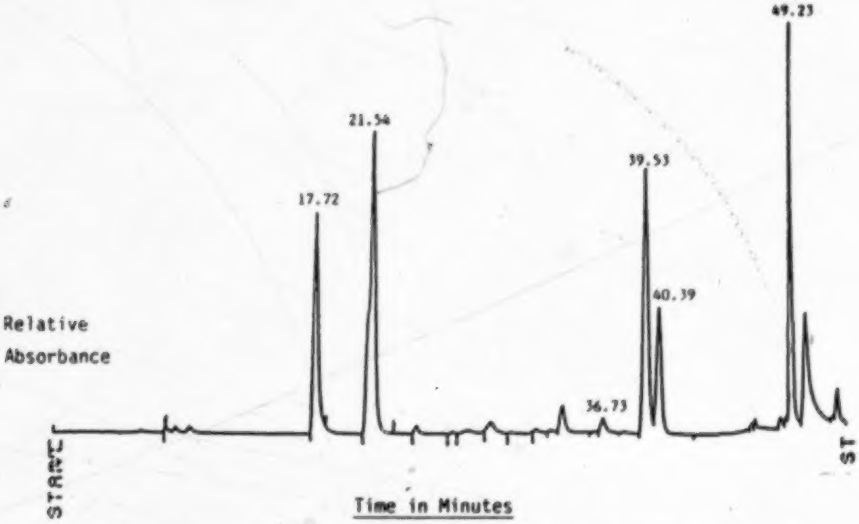
Amino Acid	Time (min.)	Area	Area/Standard	If1=2.55	Whole Number
E	7.78	604	3.32	1.39	1
S	17.34	547	2.37	0.93	1
T	20.94	369	1.82	0.71	1
G	21.24	979	3.85	1.51	1
A	24.27	732	3.13	1.23	1
R	32.13	614	2.72	1.07	1
V	32.66	810	3.10	1.22	1
L	39.19	800	3.20	1.26	1
K	49.16	693	3.18	1.25	1

Figure 36. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=6.65	Whole Number
D	7.72	2375	14.05	2.11	2
Y	25.56	1250	5.06	0.76	1
R	32.73	1291	5.71	0.86	1
PC	36.03	244	11.96	1.79	2
L	39.44	2298	9.19	1.33	1

Figure 37. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	|f|=3.21	Whole Number
S	17.72	1187	5.14	1.60	2
G + T	21.54	2137	8.41	2.62	3
C	36.73	142	6.96	2.17	2
L	39.53	1585	6.34	1.98	2
F	40.39	799	3.21	1.00	2
K	49.23	1481	6.79	2.12	2
					4

Figure 38. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 9 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.

Hydrolysis With Endoproteinase Lysine-C

The HPLC elution pattern of reduced and pyridylethylated purothionin that had been treated with EPLC for 4 hours is given in Figure 39. Eleven fractions were obtained and represented amino acids 2-45. The amino terminal lysine was not recovered.

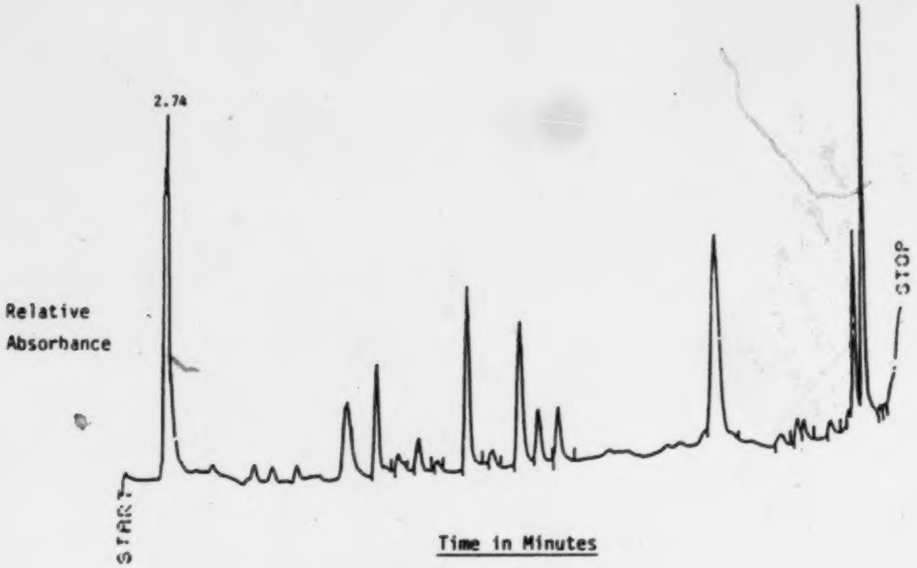
Amino acid and content of each peptide

Fraction 2 contained 1 glycine, 1 phenylalanine, and 1 lysine residue per peptide (Figure 40) and corresponded to amino acids 42-45.

Fraction 5 contained 1 serine, 1 threonine, 1 arginine, 1 valine, 1 leucine, and 1 lysine residue per peptide (Figure 41) and corresponded to amino acids 24-32. Two PC residues per peptide may exist, but the sensitivity of the printer plotter system was too low to measure the peak area when PC eluted from the column. Evidence that 3 PC residues per peptide exist is provided by fraction 4 from trypsin digestion which corresponds to amino acids 24-30 which contained 2 PC residues per peptide (Figure 31) and fraction 9 from clostripain hydrolysis which corresponded to amino acids 31-41 contained 2 PC residues.(Figure 38).

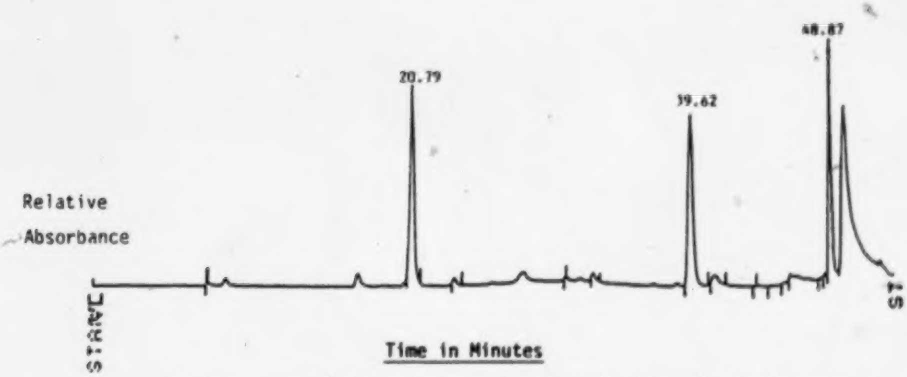
Fraction 6 contained 2 serine, 1 threonine, 1 glycine, 1 PC, 2 leucine and 1 lysine residue per peptide (Figure 42) and corresponded to amino acids 33-41. The calculated value for PC is 3.16 residues per peptide but the peak area for PC was incorrect. The given area for the peak at 41.18 is 222 and appeared significantly larger than the peak at 36.70 which had a given area of 155. In addition, the given area for the peak at 34.11 was 394 yet appears smaller than the peak at 41.18. Evidence that 1 PC residue existed in the peptide containing amino acids 33-41 was that fraction 5 from trypsin digestion contained 1 PC residue (Figure 32). Threonine and glycine did not separate completely so the area was divided equally between two amino acids.

Fraction 9 contained aspartic acid, glutamic acid, serine, threonine, glycine, alanine, tyrosine, arginine, leucine, lysine, and PC (Figure 43) and corresponded to amino acids 2-23. The area of the arginine peak indicates 5 residues per peptide but the small peak eluting just after the arginine peak may have been included in this area and only 4 residues per peptide may exist. The calculated value for threonine was 1.24 residues per peptide but threonine and glycine did not separate completely so 2 residues per peptide may exist. It was unfortunate that the trypsin or the clostripain digests containing amino acids 6-10 were not recovered. The analysis of these fractions could resolve this question.



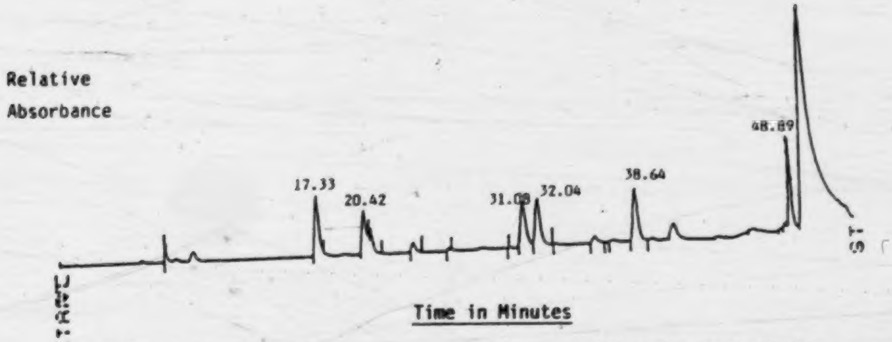
Fraction	Time
1	2.74
5	22.73
6	26.21
9	39.24

Figure 39. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. longissima* that had been treated with EPLC for 4 hours.



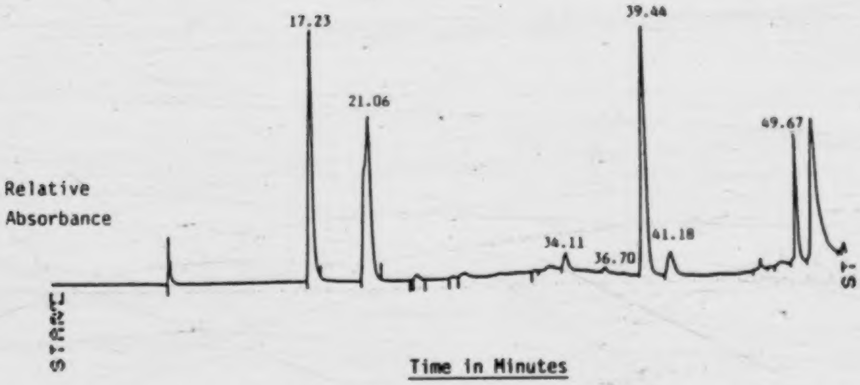
Amino Acid	Time (min.)	Area	Area/Standard	If1=3.87	Whole Number
G	20.79	984	3.87	1.00	1
F	39.62	1033	4.15	1.07	1
K	48.87	875	4.01	1.04	1

Figure 40. Results from the HPLC separation of amino acids formed by HCl hudrolysis of fraction 2 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



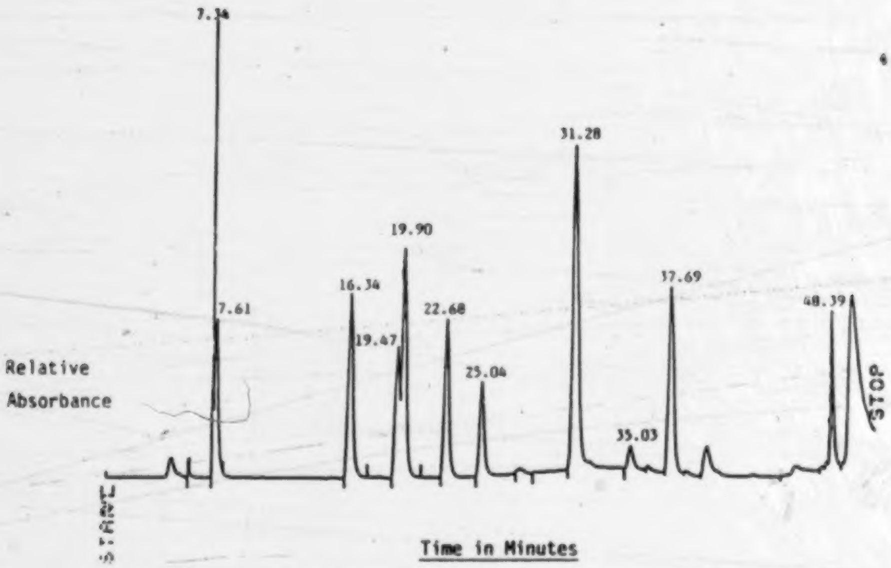
Amino Acid	Time (min.)	Area	Area/Standard	If1=1.12	Whole Number
S		266	1.15	1.03	1
T		122	0.60	0.54	1
R		272	1.07	0.96	1
V		291	1.12	1.00	1
L		346	1.38	1.23	1
K		331	1.52	1.36	1
C					

Figure 41. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=2.4	Whole Number
S	17.23	1160	5.02	2.09	2
T + G	21.06	1273	5.01	2.34	2
C	36.70	155	7.59	3.16	3
L	39.44	1489	5.96	2.48	2
K	49.67	524	2.40	1.00	1

Figure 42. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=2.59	Whole Number
D	7.34	649	3.84	1.48	2
E	7.61	603	3.31	1.28	1
S	16.34	1071	4.64	1.79	2
T	19.47	651	3.21	1.24	1
G	19.90	1338	5.27	2.03	2
A	22.68	907	3.88	1.49	1
Y	25.04	602	2.44	0.94	1
R	31.28	3148	13.93	5.38	5
C	35.03	313	15.34	5.92	5
L	37.69	1287	2.73	1.99	2
K	48.39	595	15.34	1.05	1

Figure 43. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 9 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. longissima*. Calculations of the number of amino acid residues per peptide is given above.

It is clear from the above data that the purothionin from _A. longissima_ does not resemble α_1 purothionin.

The amino acid sequence of purothionin from _A. longissima_ is given in Figure 44. It is identical to the amino acid sequence of α_2 purothionin.

K
S
C
C
5 R
T
T
L
G
10 R
N
C
Y
N
15 L
C
R
S
R
20 G
A
Q
K
L
25 C
S
T
V
C
30 R
C
K
L
T
35 S
G
L
S
C
40 P
K
G
F
P
45 K

Figure 44. The amino acid sequence of purothionin from *A. longissima*.

Extraction and Analysis of Purothionin From *A. bicornis*

The results of CMC column separation of crude purothionin from A. bicornis are given in Figures 45 and 46. After freeze drying fractions 15-17 (Figure 46), 3.1 mg. of purothionin was obtained. The results of CMC column separation of reduced and pyridylethylated purothionin is given in Figure 47. After freeze drying fractions 37-39, 2.3 mg. of protein was obtained.

Clostripain Hydrolysis

The HPLC elution pattern of reduced and pyridylethylated purothionin hydrolysed with clostripain is given in Figure 48. Eleven fractions were recovered.

Amino Acid Analysis

Fraction 3 contained 1 serine, 1 arginine, 2 PC, and 1 lysine residue per peptide (Figure 49) and corresponds to amino acids 1-5.

Fraction 4 contained one residue per peptide of serine, threonine, glycine, arginine and leucine (Figure 50) and corresponded to amino acids 6-10.

Fraction 6 contained 2 glycine, 2 alanine, and 2 PC residues per peptide and 1 glutamic acid, 1 arginine, 1 valine, 1 leucine, and 1 lysine residue per peptide (Figure 51) and corresponded to amino acids 20-30.

Fraction 7 was composed of two peptides (Figure 52). Analysis indicated that it was a complex of .92 units of a peptide which included amino acids 11-17 and .32 units of a peptide containing amino acids 18-30. After referring to the amino acid content of fraction 6, the relative amount contributed by this fraction could

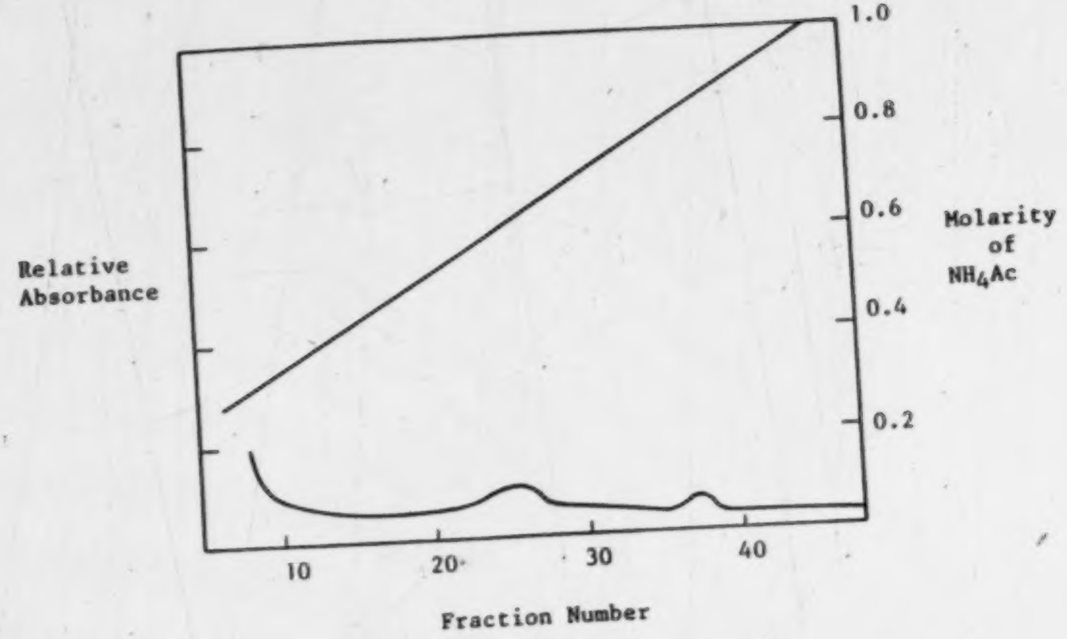


Figure 45. CMC separation of crude purothionin from _A_. _bicornis_ using a 0.3-1.1M NH_4Ac gradient, pH 5.2.

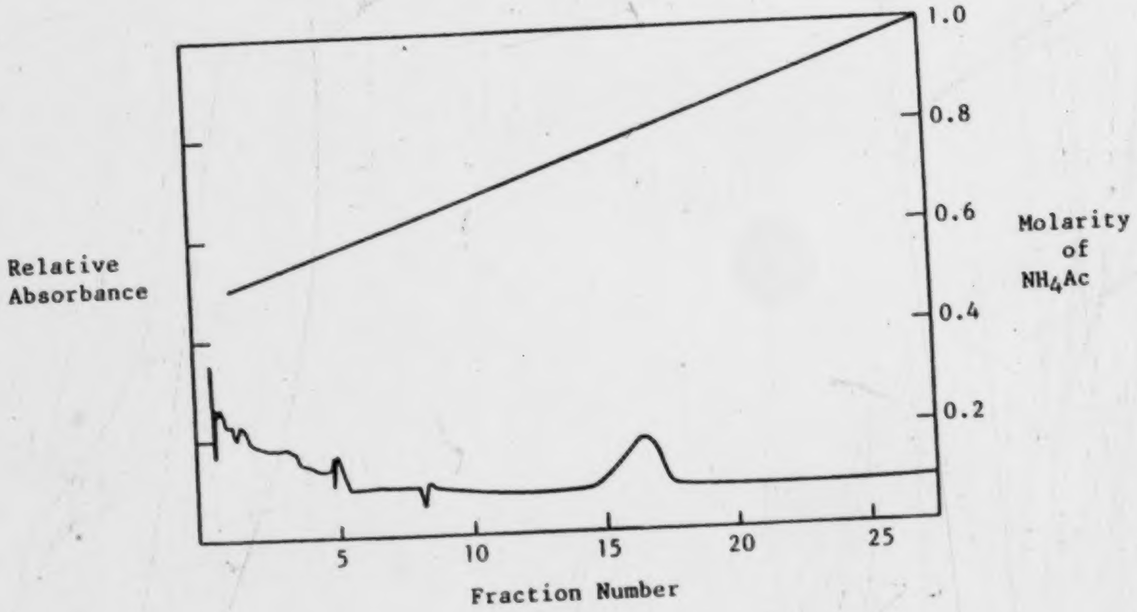


Figure 46. CMC separation of purothionin from A. bicornis using a 0.5-1.1M NH_4Ac gradient, pH 5.2.

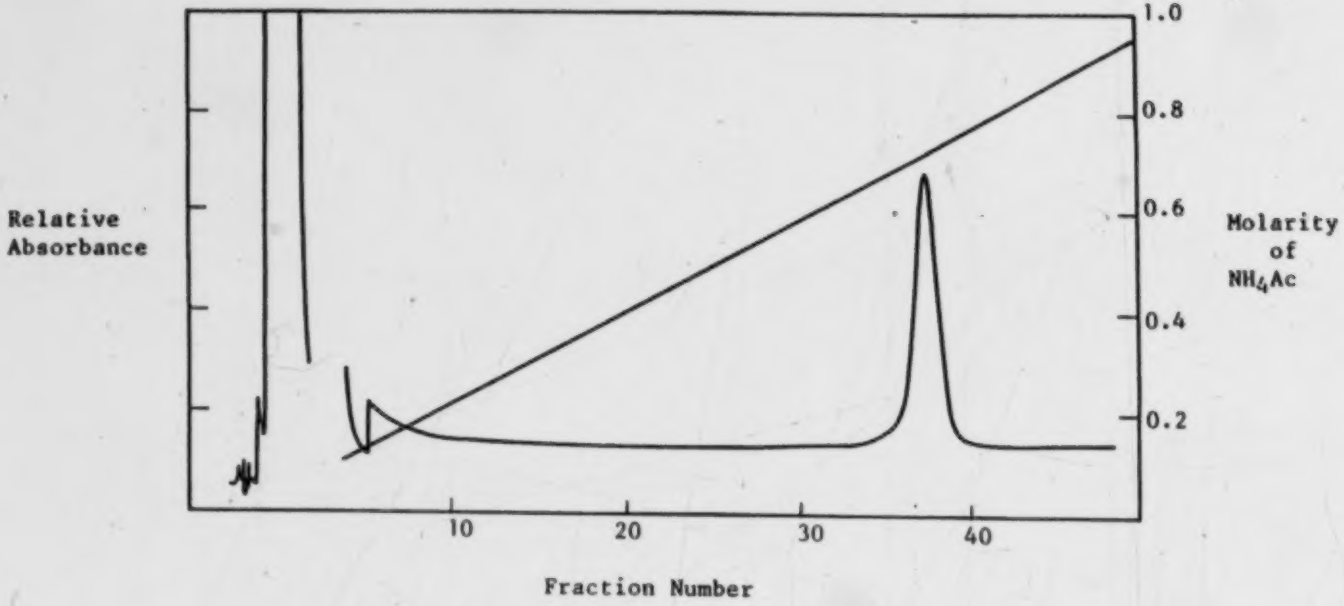
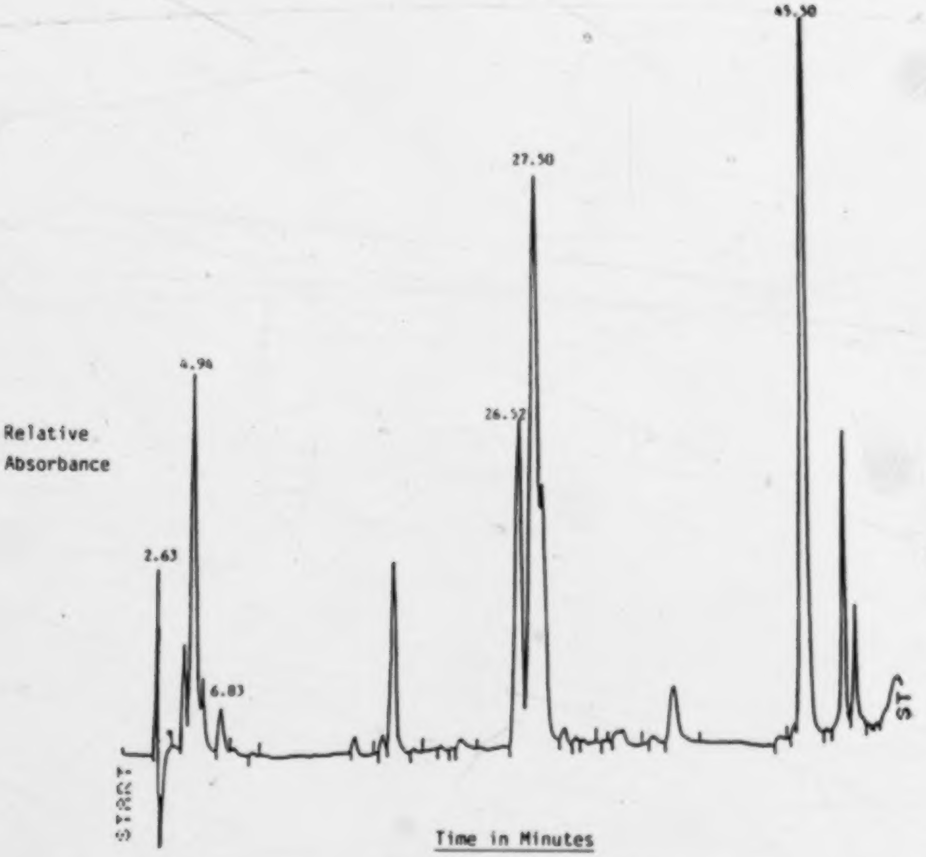
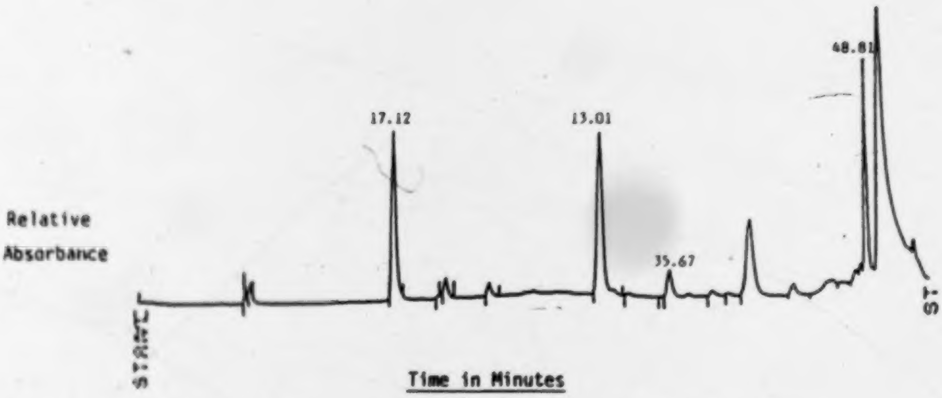


Figure 47. CMC separation of reduced and pyridylethylated purothionin from A. bicornis using a 0.1-1.1M NH_4Ac gradient, pH 5.2.



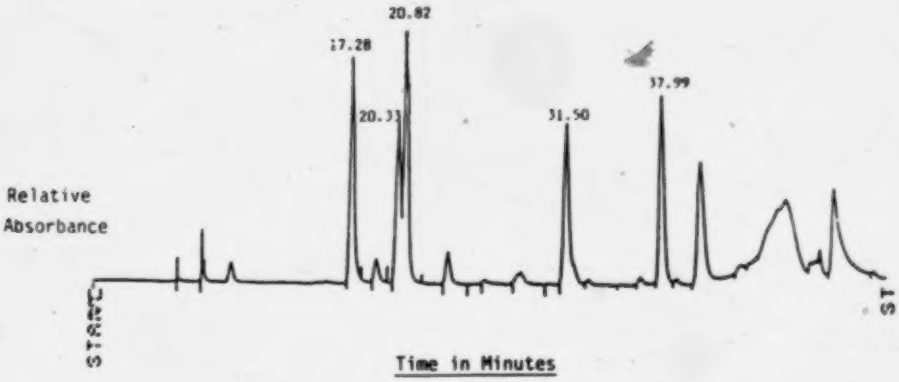
Fraction	Time (min.)
1	2.63
3	4.94
4	6.83
6	26.52
7	27.50
9	45.50

Figure 48. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. bicornis* that had been treated with clostripain for 15 minutes. Fraction number and time is given above.



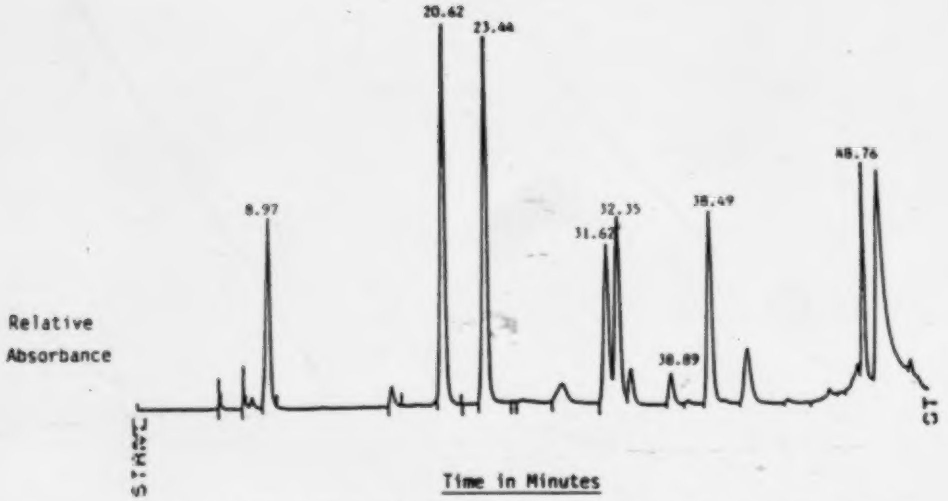
Amino Acid	Time	Area	Area/Standard	If1=3.79	Whole Number
S	17.12	875	3.79	1.00	1
R	31.01	1112	4.92	1.29	1
PC	35.67	171	8.38	2.21	2
K	48.81	918	4.21	1.11	1

Figure 49. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



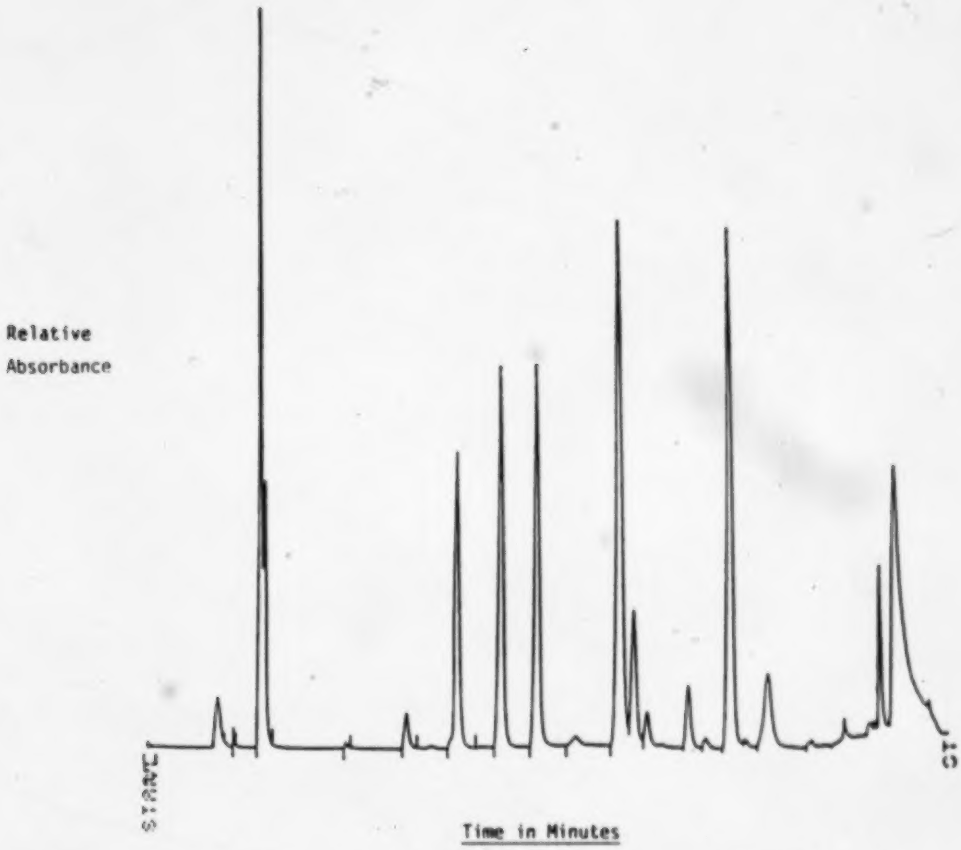
Amino Acid	Time (min.)	Area	Area/Standard	If1=4.07	Whole Number
S	17.28	1140	4.94	1.21	1
T	20.33	826	4.07	1.00	1
G	20.82	1391	5.48	1.35	1
R	31.50	1174	5.19	1.28	1
L	37.99	1236	4.94	1.21	1

Figure 50. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 4 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1-4.42	Whole Number
E	8.97	928	5.09	1.15	1
G	20.62	1989	7.83	1.77	2
A	23.44	2137	9.13	2.07	2
R	31.62	1029	4.55	1.03	1
V	32.35	1194	4.58	1.04	1
PC	35.89	211	10.34	2.34	2
L	38.49	1222	4.89	1.11	1
K	48.76	964	4.42	1.00	1

Figure 51. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Area/Standard	Corrected Value	[f]=9.08	Whole Number
D	20.13	20.13	2.22	2
E	6.28	3.05	-	-
G	6.65	0.19	-	-
A	9.28	0	-	-
Y	9.08	9.08	1.00	1
R	16.49	10.03	1.11	1
V	3.48	0.25	-	-
PC	18.97	12.51	1.38	1
L	13.05	9.82	1.08	1
K	3.23	0	-	-

Figure 52. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 7 that resulted from clostripain hydrolysis of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above. The corrected value for area/standard was obtained by multiplying 3.23 by the number of residues of each amino acid per peptide in fraction 6 and subtracting this value from the calculated area/standard from fraction 7.

be taken away from the total and the amino acids content of the peptide which included amino acids 11-17 was determined. Since only one residue of lysine occured in the peptide containing amino acids 11-30, the $\frac{\text{Area}}{\text{Standard}}$ value can be used to calculate the true peak areas of the amino acids 11-17. The calculations and values are given in Figure 52. Fraction 7 contained 2 aspartic acid, 1 tyrosine, 1 arginine, 2 PC, and 1 leucine residue per peptide. Although the calculated value for PC is 1.38 residues per peptide, analysis of this area after trypsin digestion indicated that 2 PC residues per peptide exists.

Fraction 9 contained 3 lysine, 2 serine, 2 glycine, and 2PC residues per peptide, and 1 alanine, 1 isoleucine, 1 leucine, and 1 phenylalanine residue per peptide (Figure 53) and corresponded to amino acids 31-45.

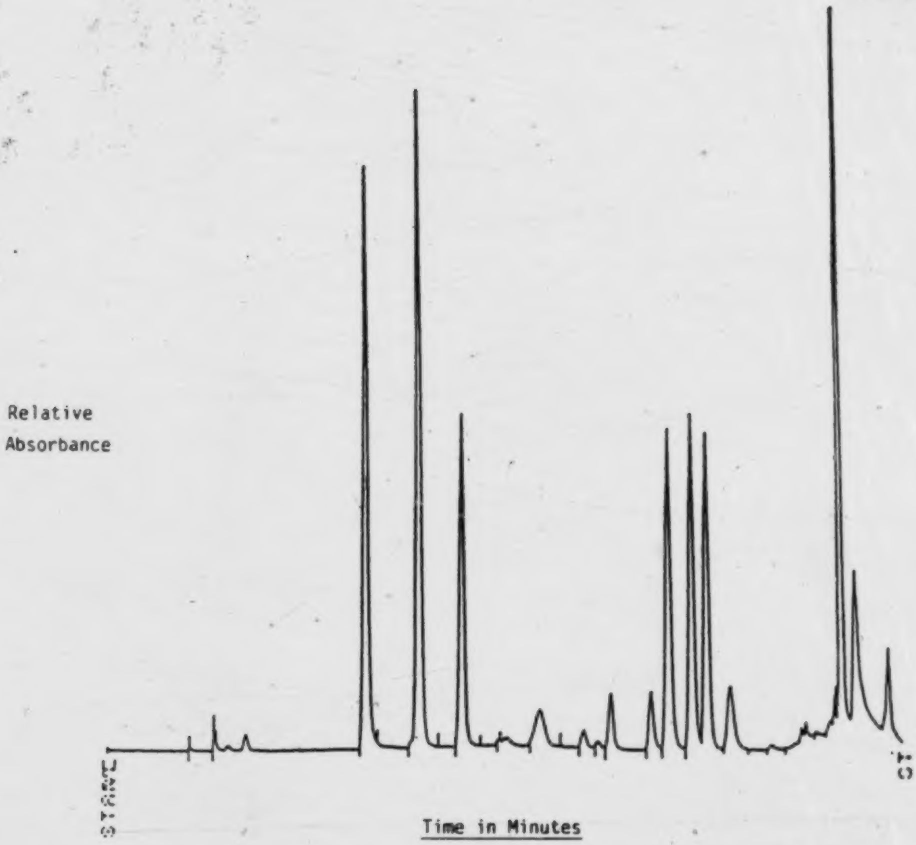
Trypsin Digestion

The HPLC elution profile of trypsin treated purothionin is given in Figure 54. Eight fractions were recovered.

Amino Acid Analysis

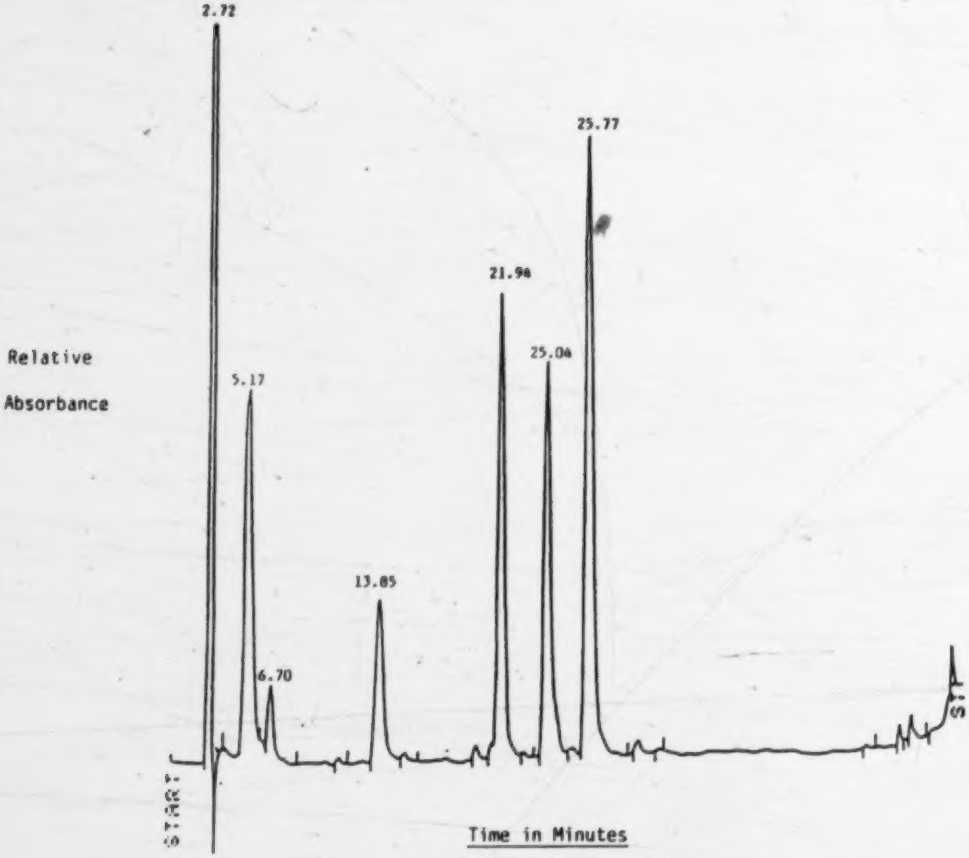
Fraction 1 contained 1 glutamic acid, 1 glycine, 2 alanine, 1 arginine, and 1 lysine residue per peptide (Figure 55) and corresponded to amino acids 18-23. Apparently trypsin did not cleave the arginine at amino acid 19.

Fraction 3 contained 1 serine, 1 threonine, 1 glycine, 1 arginine, and 1 lysine residue per peptide (Figure 56) and corresponded to amino acids 6-10.



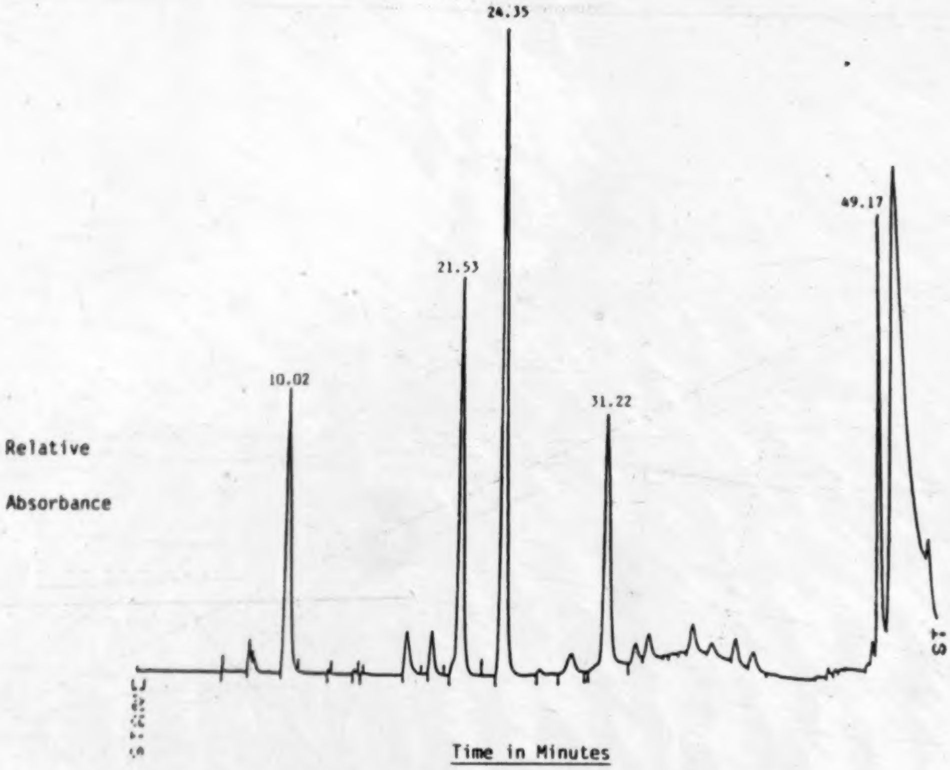
Amino Acid	Time (min.)	Area	Area/Standard	If1=7.76	Whole Number
S	17.39	3123	13.52	1.74	2
G	20.86	3550	13.98	1.80	2
A	23.67	1854	7.92	1.02	1
PC	36.24	366	17.94	2.31	2
I	37.39	2079	7.76	1.00	1
L	38.93	2063	8.25	1.06	1
F	39.93	1979	7.94	1.02	1
K	39.93	4320	19.82	2.55	3

Figure 53. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 9 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A/ bicornis*. Calculations of the number of amino acid residues per peptide is given above.



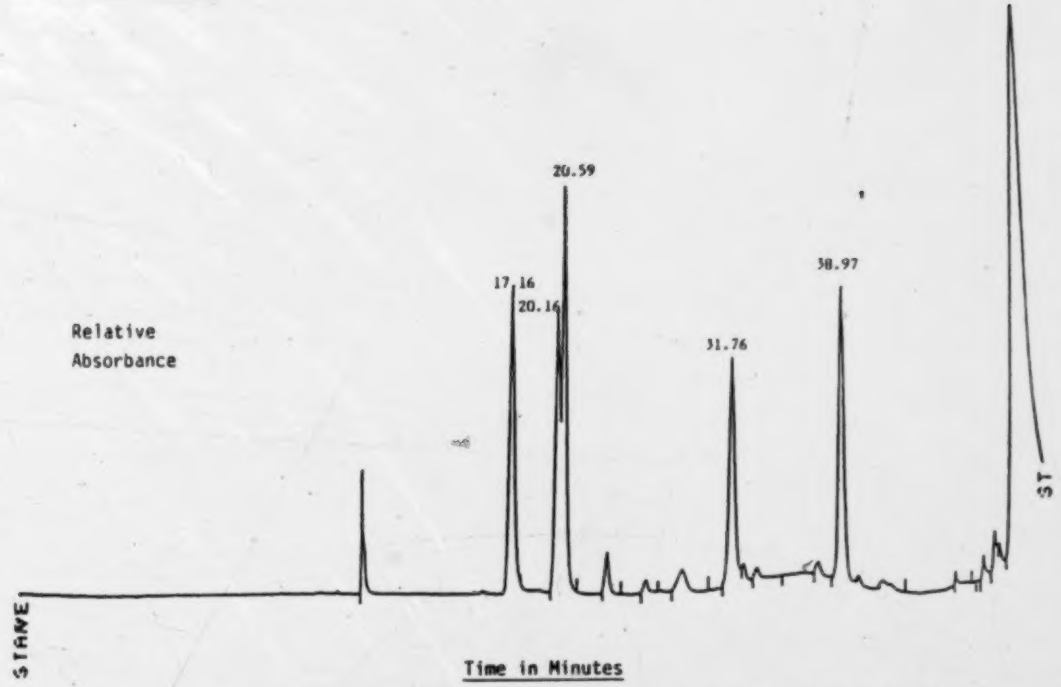
Fraction	Time (min.)
1	2.72
2	5.17
3	6.70
4	13.85
5	21.94
6	25.04
7	25.77

Figure 54. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. bicornis* that had been treated with trypsin for 1 hour. Fraction number and time are given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=9.17	Whole Number
E	10.02	1669	9.17	1.00	1
G	21.53	2282	9.98	2.98	1
A	24.35	3796	15.98	1.74	2
R	31.22	2253	9.96	1.09	1
K	49.17	1799	8.25	0.89	1

Figure 55. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 1 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



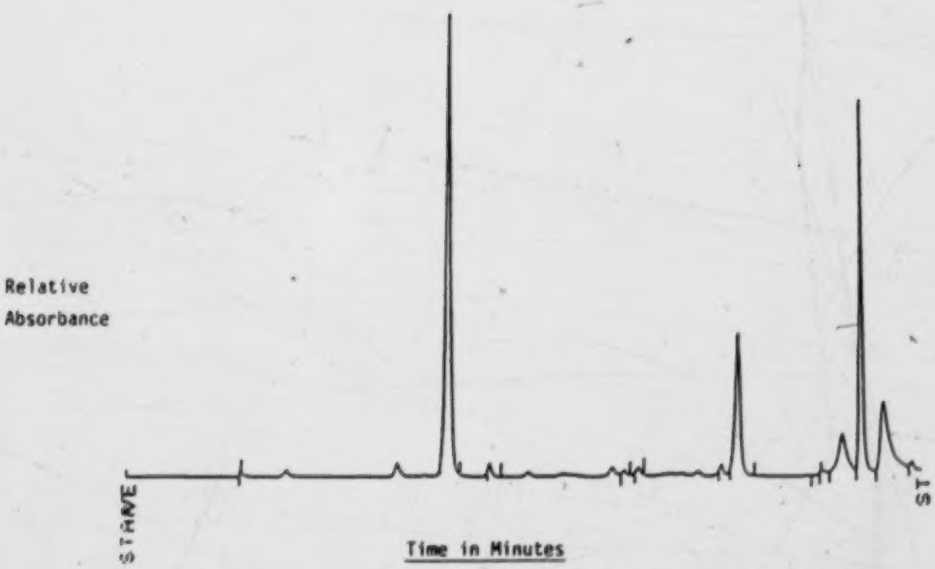
Amino Acid	Time (min.)	Area	Area/Standard	If1=2.00	Whole Number
S	17.16	1660	7.19	1.03	1
T	20.16	1421	7.00	1.00	1
G	20.59	2150	8.47	1.21	1
R	31.76	1620	7.17	1.02	1
L	38.97	1979	7.91	1.13	1

Figure 56. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.

Fraction 4 contained glycine, phenylalanine, and lysine (Figure 57) and corresponded to amino acids 42-45. Unfortunately, the OPA solution used was too old and unreliable , hence relative amounts could not be determined. However, from the analysis of fraction 9, after clostripain treatment, it is clear that this fragment represented amino acids 42-45.

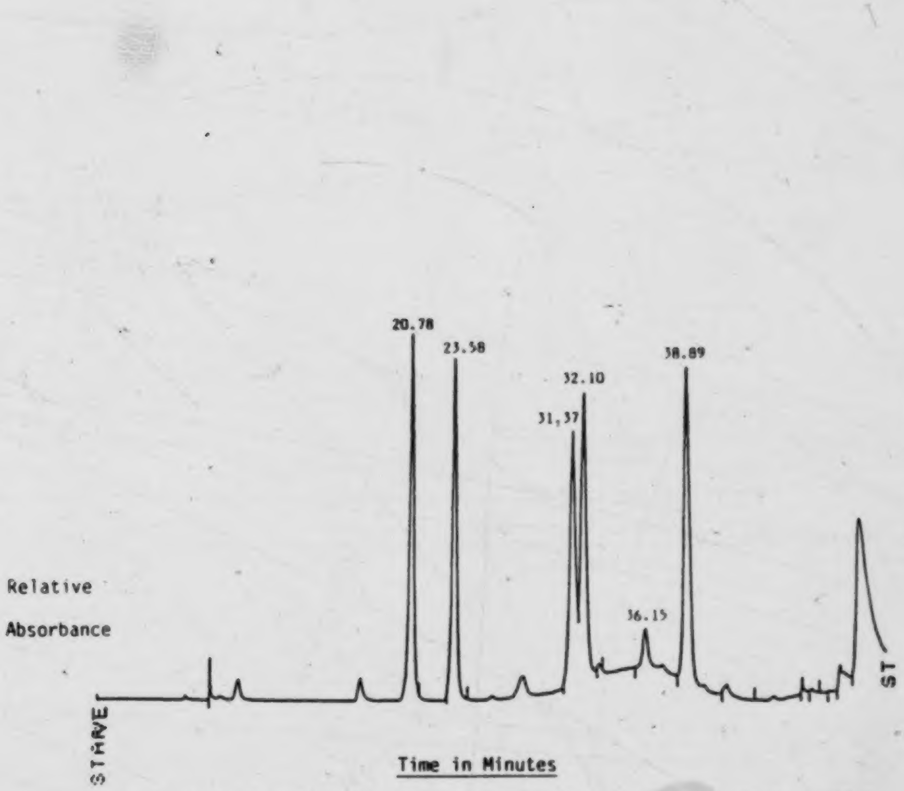
Fraction 5 contained 3 residues of PC and 1 residue per peptide of glycine, alanaine, arginine, valine, and leucine (Figure 58) which corresponded to amino acids 24-30. The calculated value for PC is 3.84, which may be an overestimate since the area may have included the small peak that occurs just after the peak at 36.15 minutes. In addition, fraction 6 from clostripain hydrolysis which included amino acids 20-30 contained only 2 PC residues per peptide.

Fraction 6 included a minor fraction that appears as a shoulder at the end of the main peak (Figure 59). The minor fraction included aspartic acid and glutamic acid and was disregarded in the calculations. The isoleucine, leucine, and PC areas are overestimated. The baseline change in the area where these amino acids eluted from the column resulted in an overestimate of the true area. In addition, the paper in the printer plotter which monitored the relative fluorescence at 220 nm was caught up in the machine and consequently the elution profile given is from the printer plotter which monitored at 254 nm. The main portion of fraction 6 contains 2 serine, 1 glycine, 1 alanine, 1 PC, 1 leucine, 1 isoleucine, and 1 lysine residue per peptide and represents amino acids 33-41.



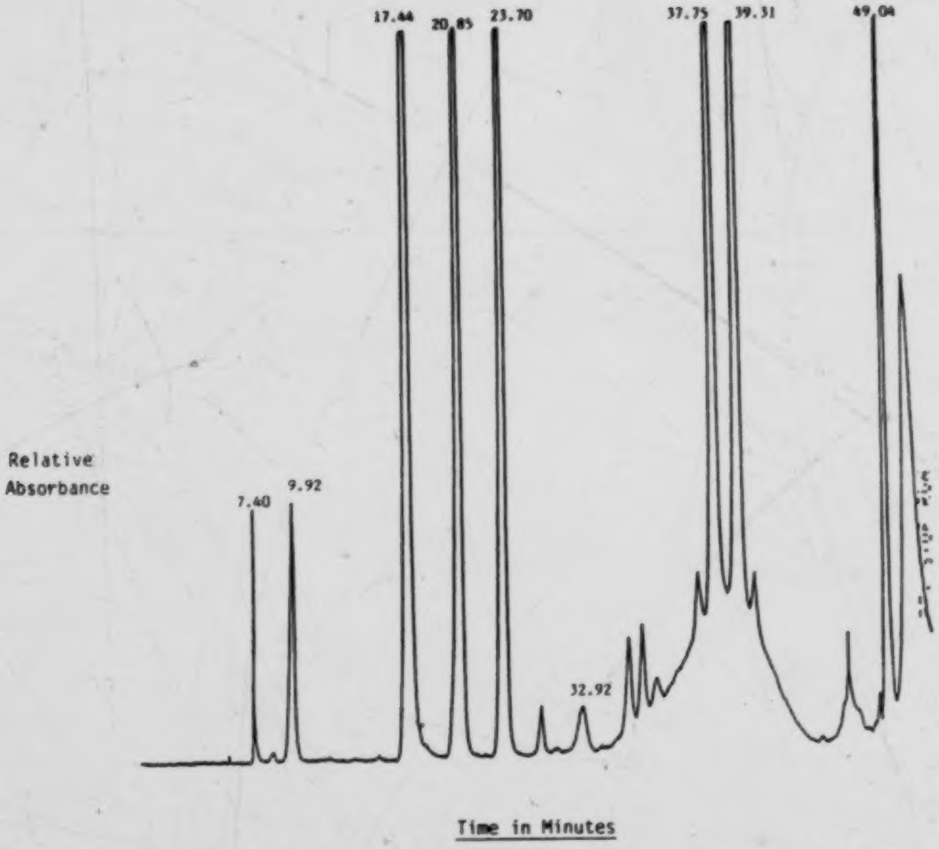
Amino Acid	Time (min.)	Area	Area/Standard
G	21.46	2509	9.88
F	40.85	924	3.71
K	49.06	1453	6.67

Figure 57. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 4 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



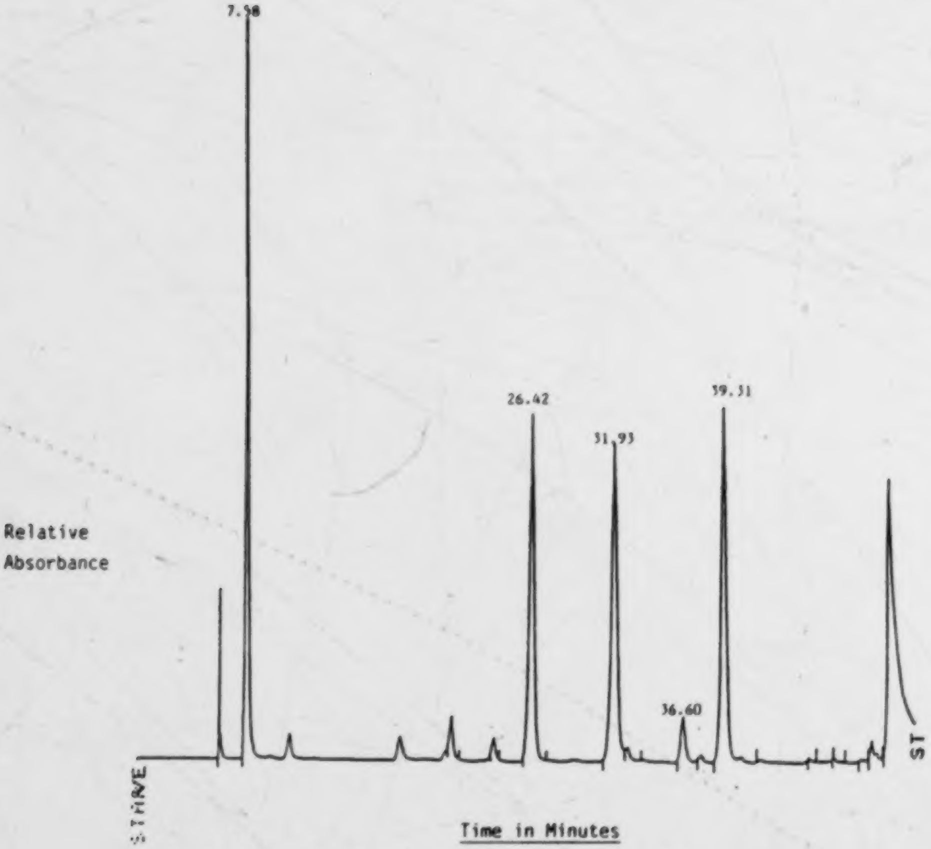
Amino Acid	Time (min.)	Area	Standard Area	If1=6.43	Whole Number
G	20.78	1634	6.43	1.00	1
A	23.58	1860	7.95	1.24	1
R	31.37	1714	7.58	1.18	1
V	32.10	1879	7.19	1.11	1
PC	36.15	501	24.56	3.82	4
L	38.89	2074	8.29	1.29	1

Figure 58. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard
D	7.40	103	0.61
E	9.92	365	2.01
S	17.44	3320	14.37
G	20.85	2160	8.50
A	23.70	2268	9.69
PC	32.92	301	14.75
I	37.75	3479	12.98
L	39.31	3528	14.11
K	49.04	1656	7.59

Figure 59. Results from the HPLC separation of maino acids formed by HCl hydrolysis of fraction 6 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=8.22	Whole Number
D	7.58	2817	16.67	2.02	2
Y	26.47	2031	8.22	1.00	1
R	31.93	2177	9.63	1.17	1
PC	36.60	267	13.09	1.60	2
L	39.31	2326	9.30	1.13	1

Figure 60. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 7 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.

Fraction 7 contained 2 aspartic acid, 2 PC, 1 tyrosine, 1 arginine, and 1 leucine residue per peptide (Figure 60) and represented amino acids 11-17.

Endoproteinase Lysine-C Hydrolysis

The HPLC elution profile EPLC hydrolysis of purothionin from *A. bicornis* is given in Figure 61. Eleven fractions were obtained.

Amino Acid Analysis

Unfortunately, the relative amounts of each amino acid in fractions 3, 6, and 8 could not be calculated. However, by comparing the amino acid content from trypsin and clostripain generated fragments, relative amino acid contents could be estimated.

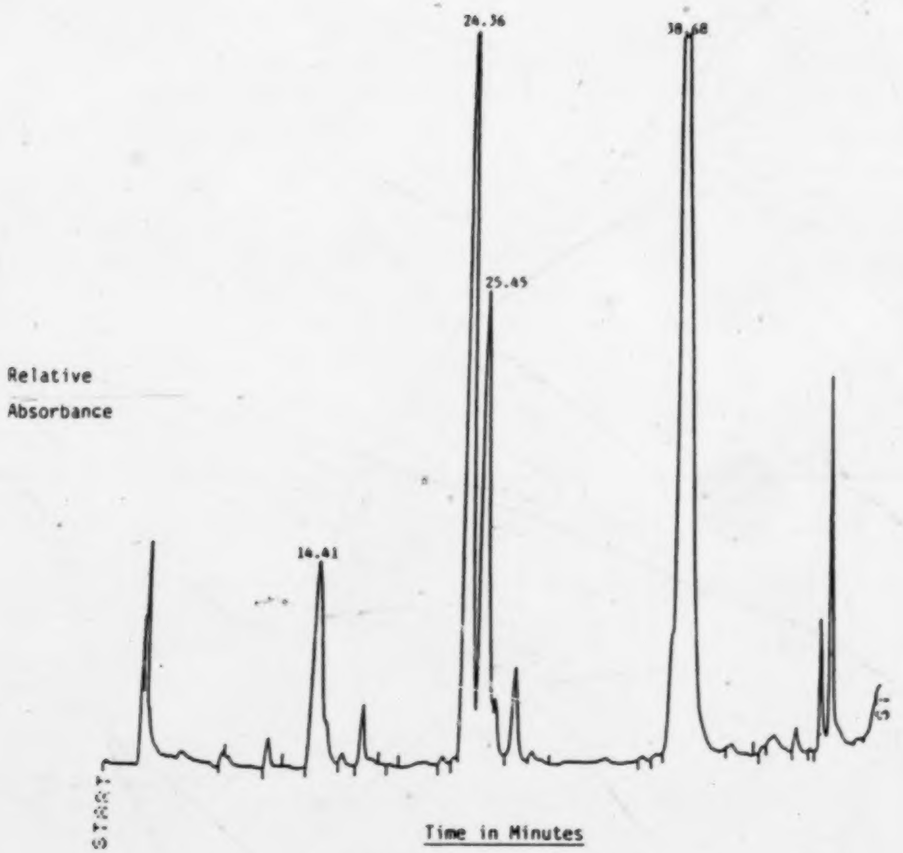
Fraction 3 contained glycine, phenylalanine and lysine (Figure 62) and corresponded to amino acids 42-45.

Fraction 5, which was analysed with fresh OPA contained 3 PC, 1 glycine, 1 alanine, 1 arginine, 1 valine, 1 leucine, and 1 lysine residue per peptide (FIgure 63) and corresponded to amino acids 24-32.

Fraction 6 contained serine, glycine, alanine, isoleucine, leucine, and lysine (Figure 64) and corresponded to amino acids 33-41.

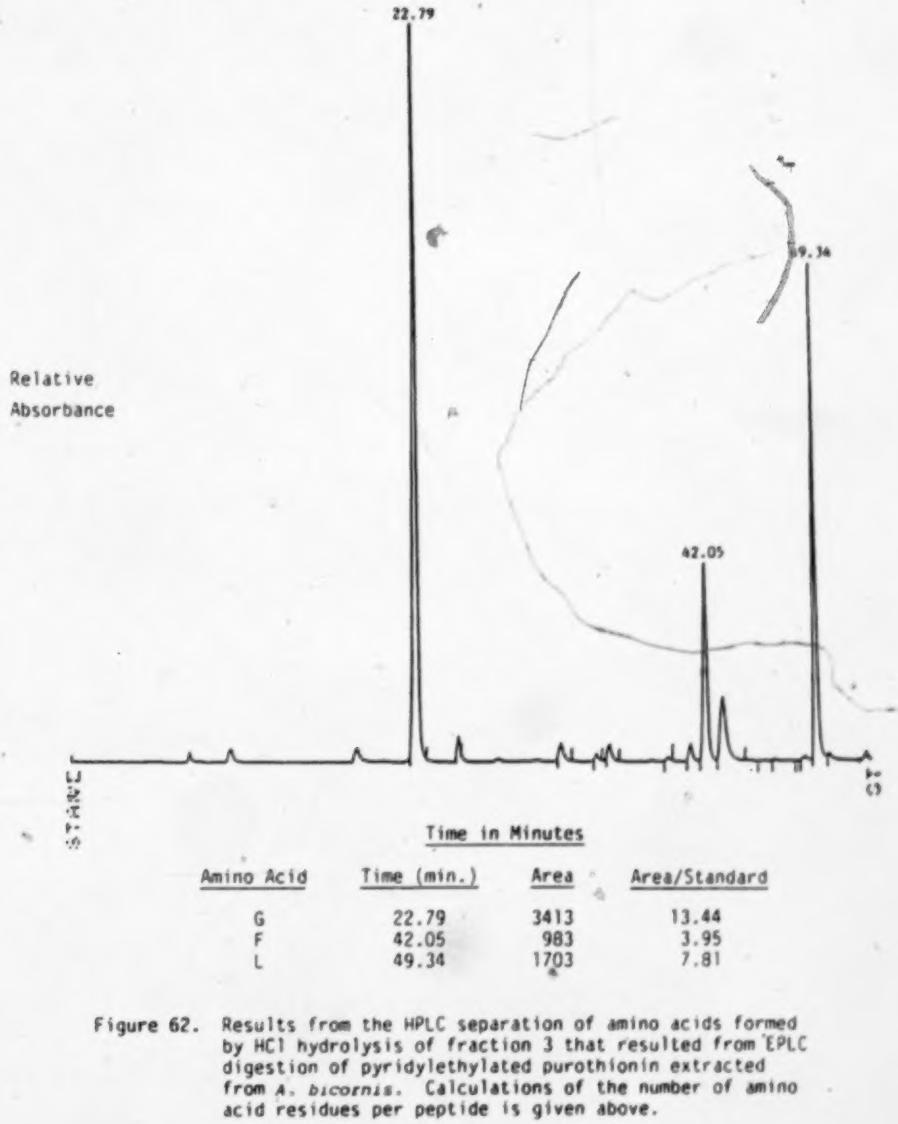
Fraction 8 contained aspartic acid, glutanic acid, serine, threonine, glycine, alanine, tyrosine, arginine, PC, leucine and lysine (Figure 65) and corresponded to amino acids 2-23.

The amino acid sequence of purothionin isolated from *A. bicornis* is given in Figure 66. It differs from α_1-purothionin by a change from serine to alanine at positions 34, 35, or 38.



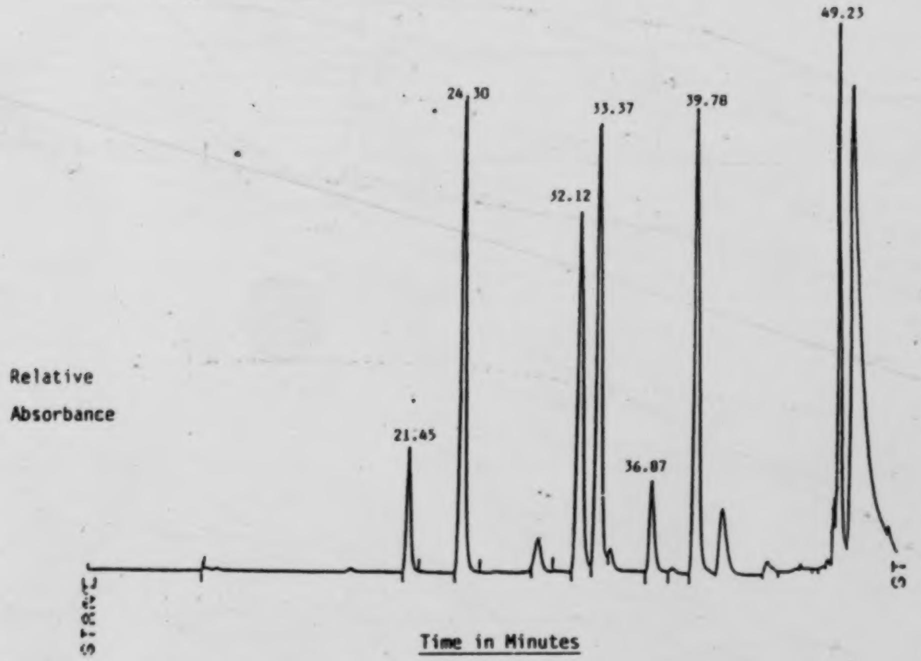
Fraction	Time (minutes)
3	14.41
5	24.36
6	25.45
8	38.68

Figure 61. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. bicornis* that had been treated with EPLC for 1 hour. Fraction number and elution time is given above.



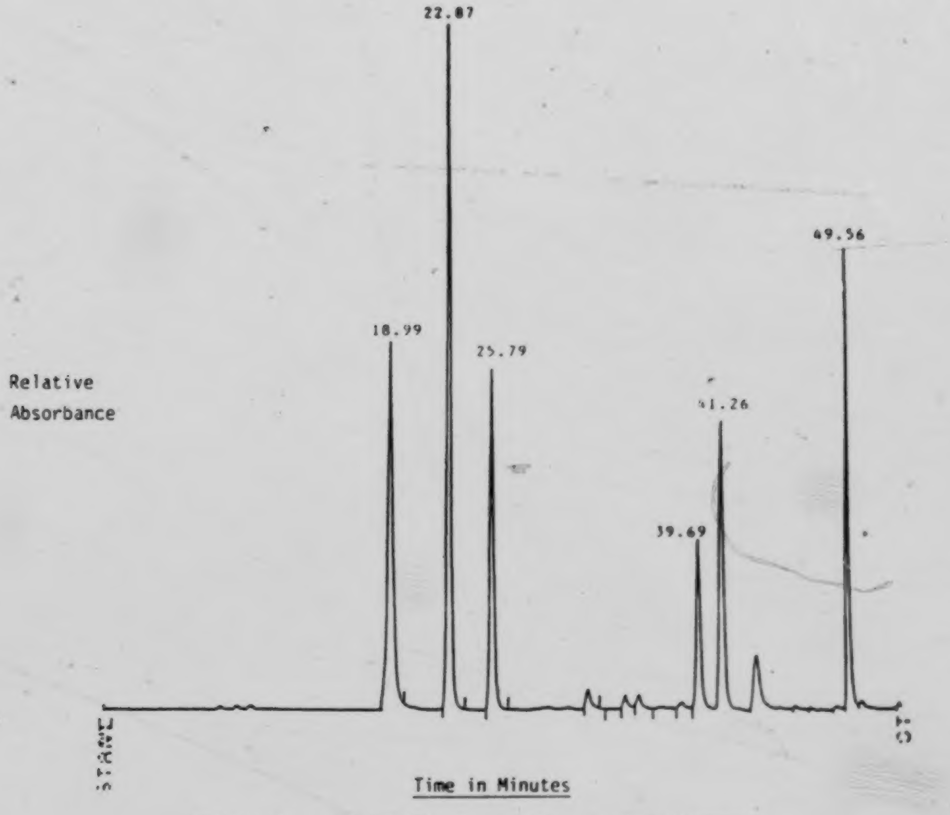
Amino Acid	Time (min.)	Area	Area/Standard
G	22.79	3413	13.44
F	42.05	983	3.95
L	49.34	1703	7.81

Figure 62. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



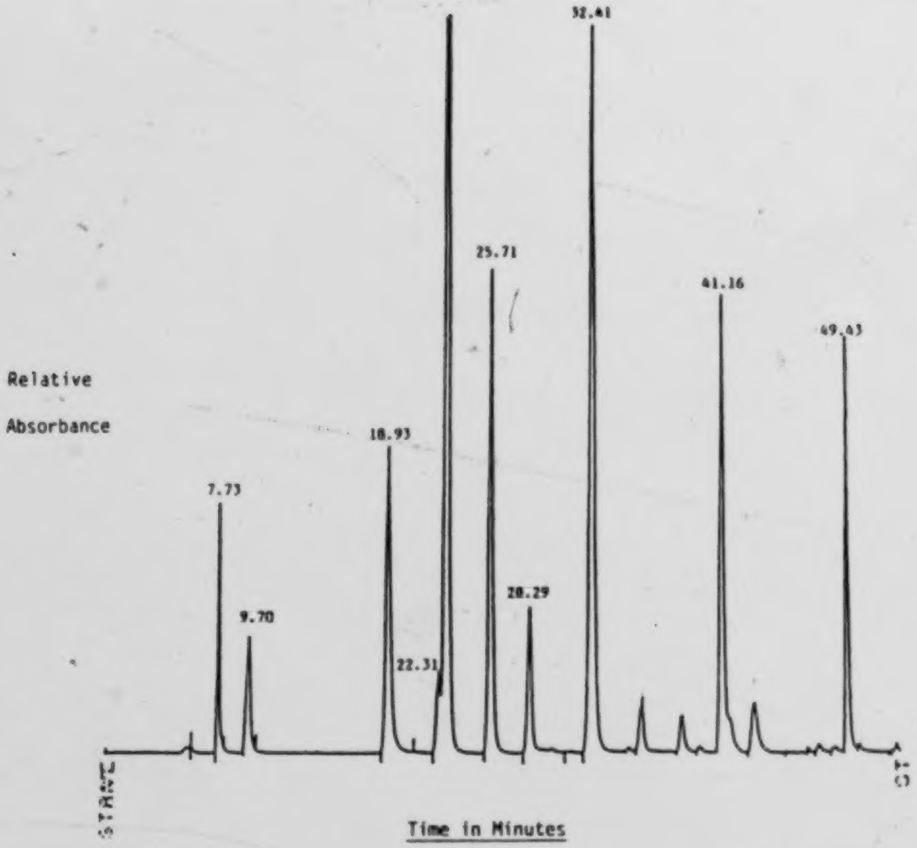
Amino Acid	Time (min.)	Area	Area/Standard	If1=9.42	Whole Number
G	21.45	2565	10.09	1.07	1
A	24.38	2677	11.44	1.21	1
R	32.12	2417	10.69	1.14	1
V	33.37	2766	10.59	1.12	1
PC	36.87	572	28.04	2.98	3
L	39.78	2845	11.38	1.21	1
K	49.23	2045	9.42	1.00	1

Figure 63. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard
S	18.99	2287	9.90
G	22.87	3239	12.75
A	25.79	1678	7.17
I	39.69	956	3.57
K	41.26	1564	6.26
L	49.56	1581	7.25

Figure 64. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard
D	7.73	650	3.85
E	9.70	549	3.02
S	18.93	1843	7.98
T	22.31	371	1.83
G	22.77	5979	23.54
A	25.71	2447	10.05
Y	28.29	845	3.42
R	32.41	4496	19.89
PC	38.47	253	12.40
L	41.16	2608	10.43
K	49.43	1421	6.52

Figure 65. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 8 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. bicornis*. Calculations of the number of amino acid residues per peptide is given above.

K
S
C
C
5 R
S
T
L
G
10 R
N
C
Y
N
15 L
C
R
A
R
20 G
A
Q
K
L
25 C
A
G
V
C
30 R
C
K
I
* S
35* S
G
L
* S
C
40 P
K
G
F
P
45 K

* One of these three serine residues is alanine.

Figure 66. The amino acid sequence of purothionin isolated from *A. bicornis*.

Extraction and Analysis of Purothionin From *A. searsii*

The results of CMC column separation of crude purothionin from A. searsii are given in Figures 67 and 68. After freeze drying fractions 14-17 (Figure 68), 2.0 mg of purothionin was obtained. The results of CMC column separation of reduced and pyridylethylated purothionin is given in Figure 69. After freeze drying fractions 36-39, 1.7 mg of protein was obtained.

Clostripain Hydrolysis

The HPLC elution pattern of purothionin cleaved with clostripain is given in Figure 70. Eight fractions were recovered.

Amino Acid Analysis

Fraction 1 contained 1 serine and 1 arginine residue per peptide (Figure 71) and corresponded to amino acids 18 and 19. The peaks at 45.79 and 49.72 do not represent any amino acid.

Fraction 3 contained 2 PC, 1 lysine, 1 serine, and 1 arginine residue per peptide (Figure 72) and represented amino acids 1-5. The calculated values for PC and lysine were 2.78 and 1.70 respectively. These values are overestimations since the rise in the baseline caused the printer plotter to overestimate the peak areas.

Fraction 5 contained 1 serine, 1 threonine, 1 glycine, 1 arginine, and 1 lysine residue per peptide (Figure 73) and represented amino acids 6-10.

Fraction 6 contained 2 PC, 2 glycine, 2 alanine, 1 glutamic acid, 1 arginine, 1 valine, 1 leucine, and 1 lysine residue per peptide (Figure 74) and corresponded to amino acids 20-30. The calculated value for PC is 2.67 residues per peptide. It is felt that there are only

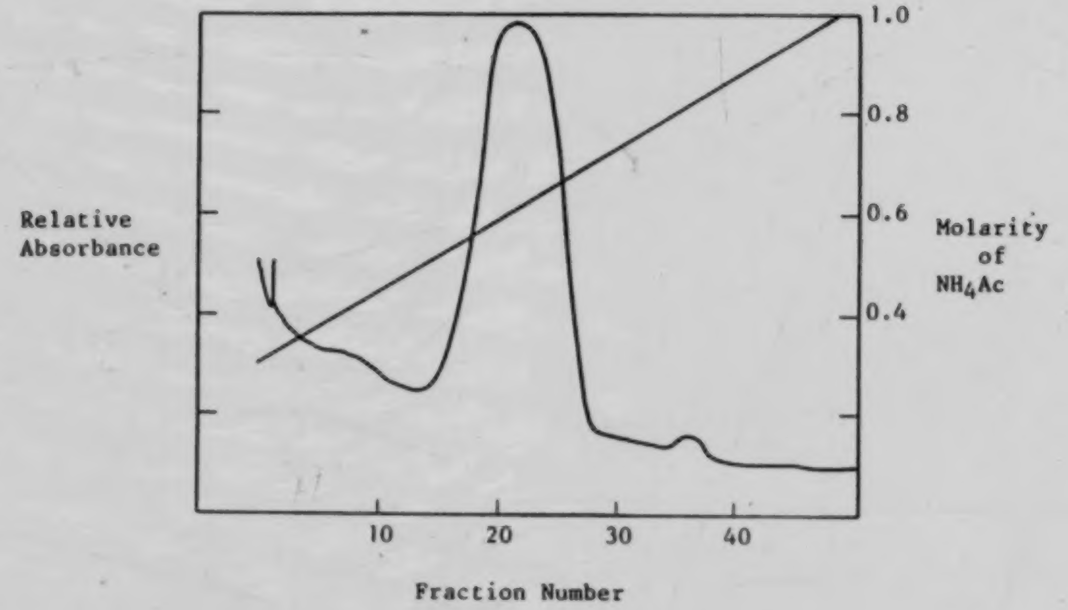


Figure 67. CMC separation of crude purothionin from A. searsii using a 0.3-1.1M NH$_4$Ac gradient, pH 5.2.

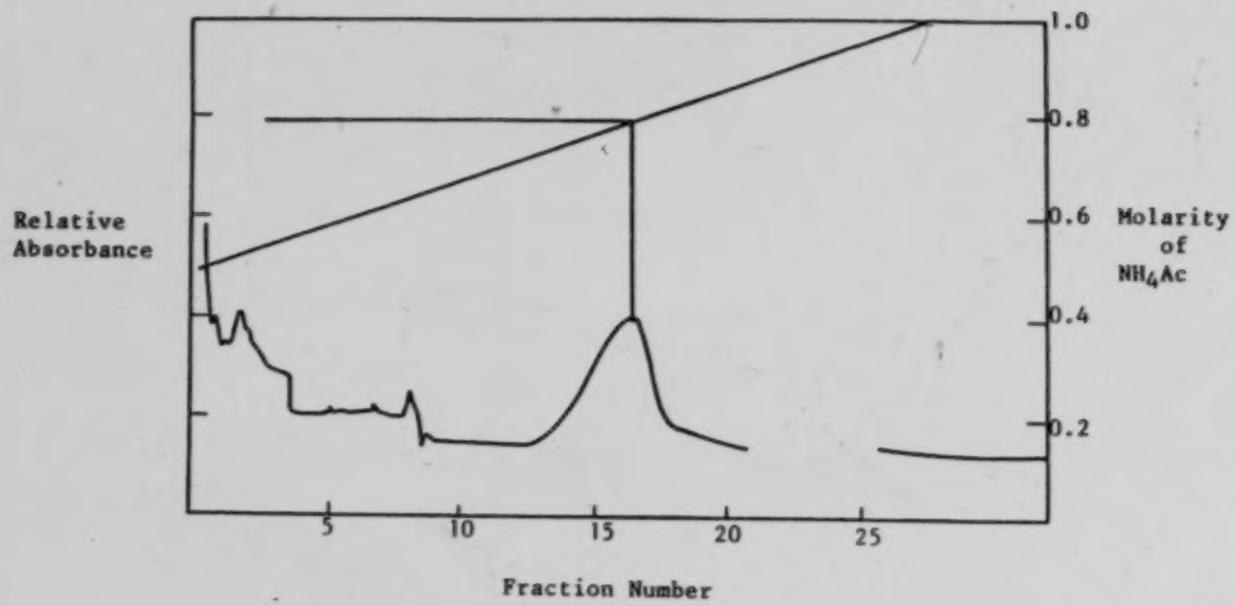


Figure 68. CMC separation of purothionin from A. searsii using a 0.5-1.1M NH_4Ac gradient, pH 5.2.

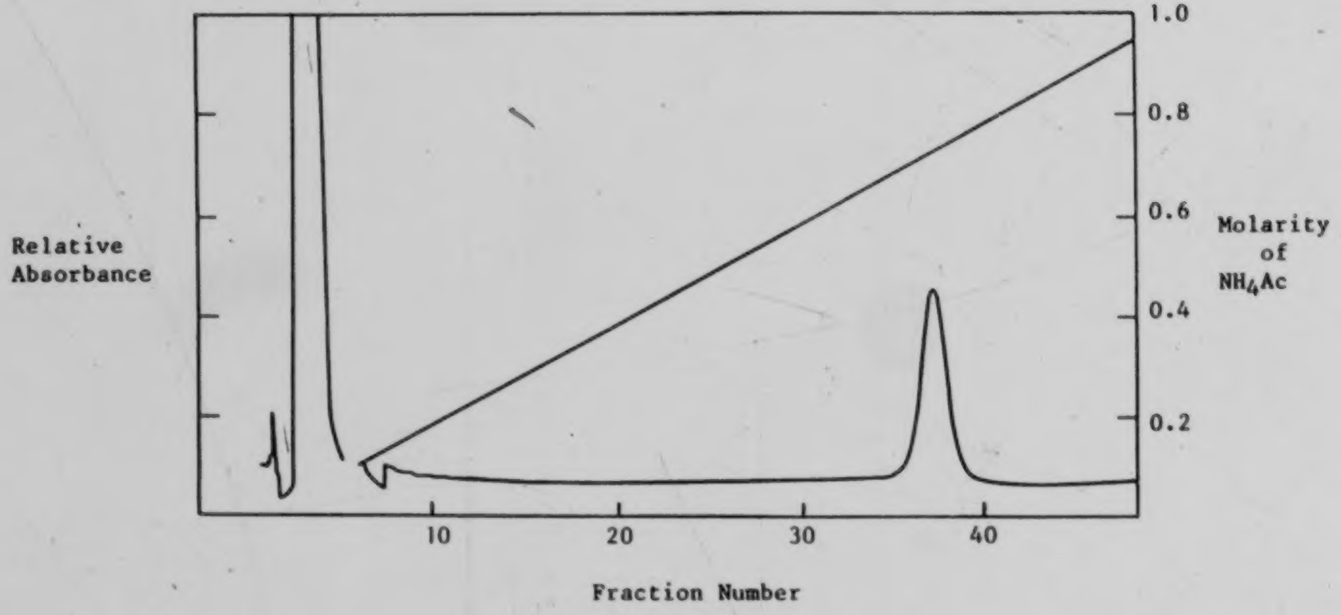


Figure 69. CMC separation of reduced and pyridylethylated purothionin from *A. searsii* using a 0.1-1.1M NH$_4$Ac gradient, pH 5.2.

2.0 PC residues per peptide since all amino acids in the peptide that includes amino acids 20 to 30 are accounted for if there are 2 PC residues per peptide.

Fraction 7 contained 2 aspartic acid, 2 PC, 1 tyrosine, 1 arginine, and 1 leucine residue per peptide (Figure 75) and corresponded to amino acids 11-17. The calculated value for PC is 1.37 residues per peptide. It appears that 2 PC residues per peptide existed. If only one PC residue existed in this peptide, the overall physical structure of the protein would be changed so the deletion of a cystine residue is unlikely.

Fraction 8 contains 3 serine, 2 glycine, 2 PC, 1 isoleucine, 1 leucine, 1 phenylalanine, and 3 lysine residues per peptide (Figure 76) and corresponded to amino acids 31-45.

Trypsin Hydrolysis

The HPLC elution pattern of purothionin cleaved with trypsin is given in Figure 77. Seven fractions were recovered.

Amino Acid Analysis

Fraction 1 contains 1 glutamic acid, 1 serine, 1 glycine, 1 alanine, 1 arginine, and 1 lysine residue per peptide (Figure 78) and corresponded to amino acid 18-23. The calcualted values for arginine was 2.69 and lysine was 2.16 residues per peptide. It was felt that these are overestimations since the baseline rises significantly at a time when arginine and lysine eluted from the column.

Fraction 2 contains 1 serine, 1 arginine, 2 PC, and 1 lysine residue per peptide (Figure 79) and corresponds to amino acids 1-5.

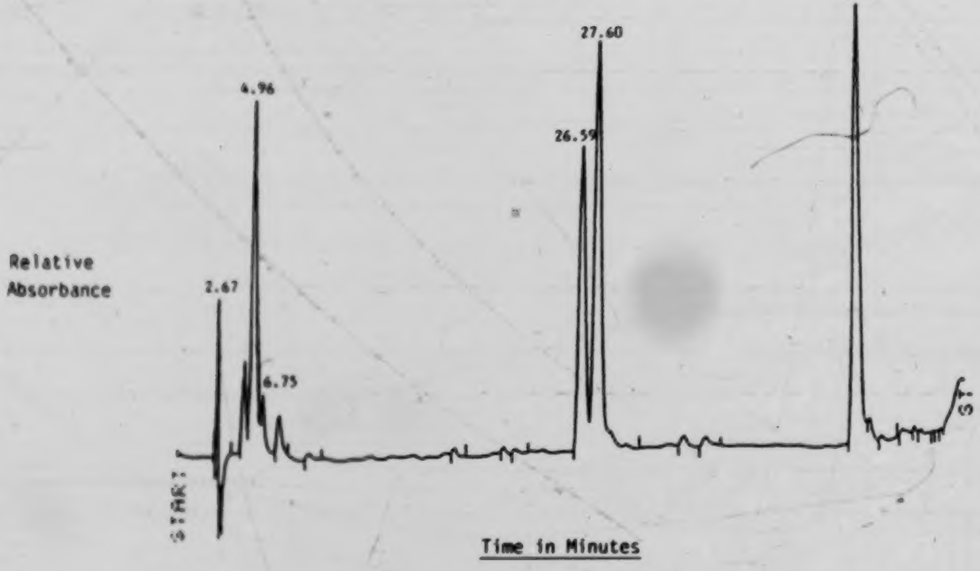
Fraction 3 contained serine, threonine, glycine, arginine, and leucine (Figure 80). Unfortunately, the relative amounts of each amino acid could not be calculated since the OPA used was too old. However, a peptide which contained 1 serine, 1 threonine, 1 leucine, 1 glycine, and 1 arginine was expected and corresponds to amino acids 6-10.

Fraction 4 contained glycine, pheylalanine, and lysine (Figure 81) and correspnded to amino acids 42-45. The relative amounts of each amino acid could not be calcualted because the OPA was too old. However, the only peptide expected that contained glycine, phenylalanine, and lysine corresponded to amino acids 42-45.

Fraction 5 contained glycine, alanine, arginine, valine, PC, and leucine residues (Figure 82) and corresponded to amino acids 24-30. Although the relative amounts of each amino acid could not be calculated, the only peptide expected that contained the above amino acids corresponded to amino acids 24-30.

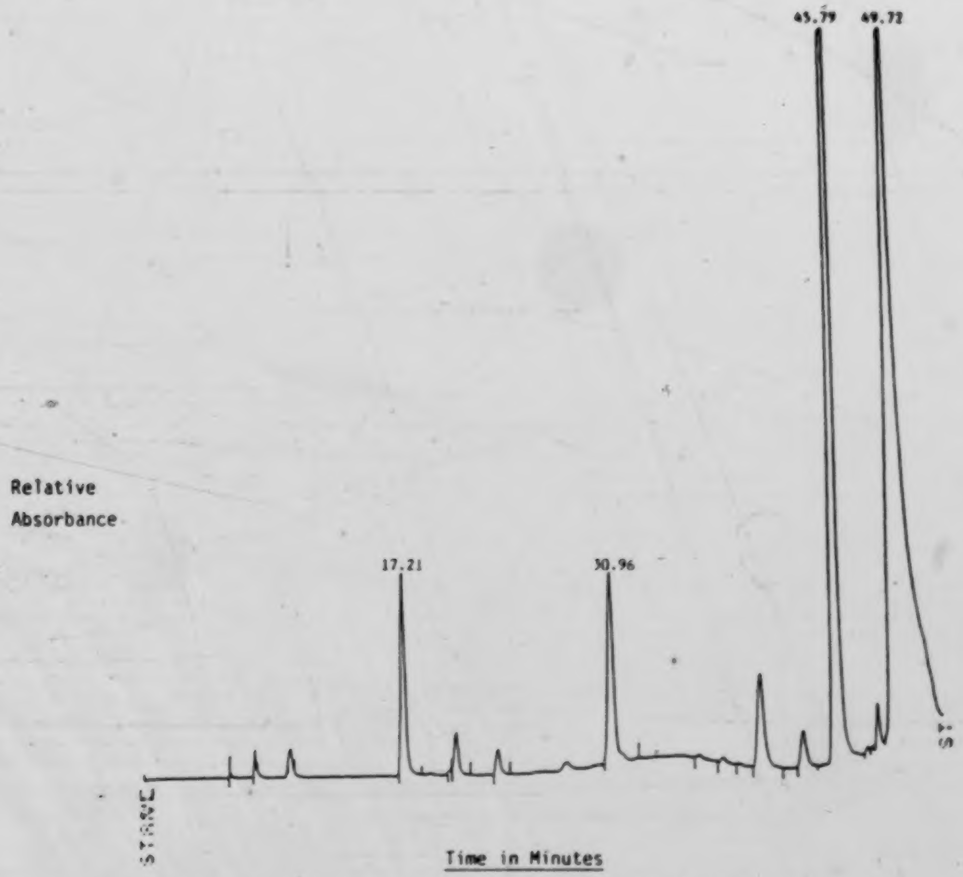
Fraction 6 contained 3 serine, 1 glycine, 1 PC, 1 isoleucine, 1 leucine, and 1 lysine residue per peptide (Figure 83) and corresponded to amino acids 33-41. The calculated value for serine residue per peptide was 2.41 but it was felt that this may be a slight underestimation since fraction 8 from clostripain digestion which included amino acids 31-45 contained 3 serine residues. The number of PC residues could not be calculated since the rise in the baseline prevented a distinct PC elution peak at 36.13.

Fraction 7 contained aspartic acid, tyrosine, arginine, PC, and leucine (Figure 84) and corresponded to amino acids 11-17. The number of each amino acid per peptide could not be calculated because the OPA was too old.



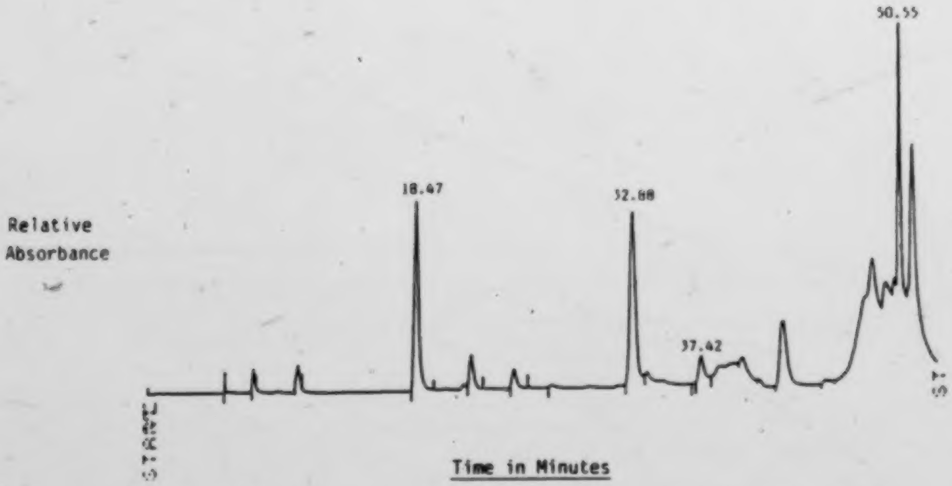
Fraction	Time (min.)
1	2.67
3	4.96
5	6.75
6	26.59
7	27.60
8	44.85

Figure 70. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. searsii* that had been treated with clostripain for 15 minutes. Fraction number and elution time is given above.



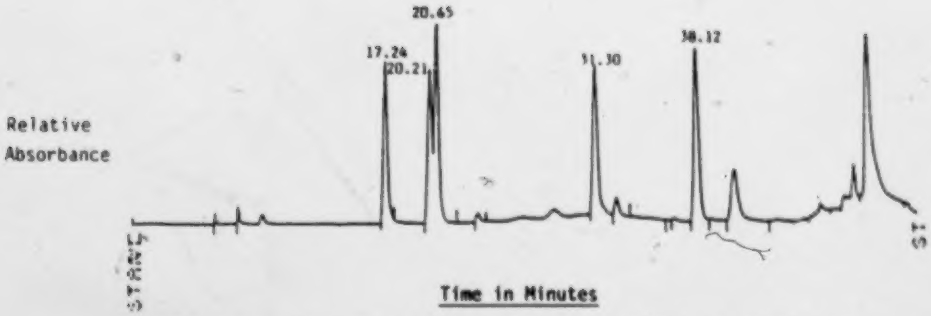
Amino Acid	Time (min.)	Area	Area/Standard	[f]=4.72	Whole Number
S	17.21	1090	4.72	1.00	1
R	30.96	1329	5.88	1.25	1

Figure 71. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 1 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



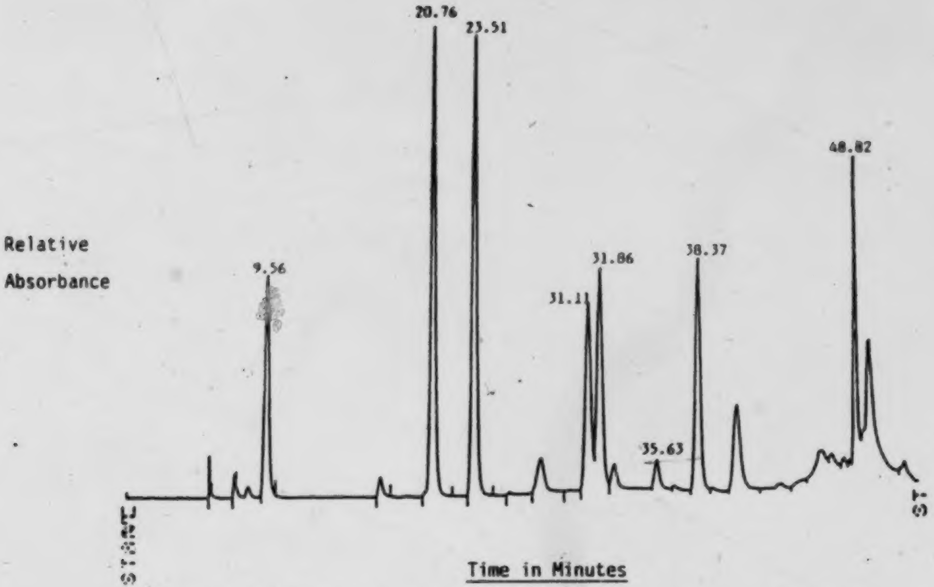
Amino Acid	Time (min.)	Area	Area/Standard	If1=4.52	Whole Number
S	18.47	1044	4.52	1.00	1
R	32.88	1355	5.99	1.32	1
PC	37.42	256	12.55	2.78	3
K	50.55	1679	7.70	1.70	2

Figure 72. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



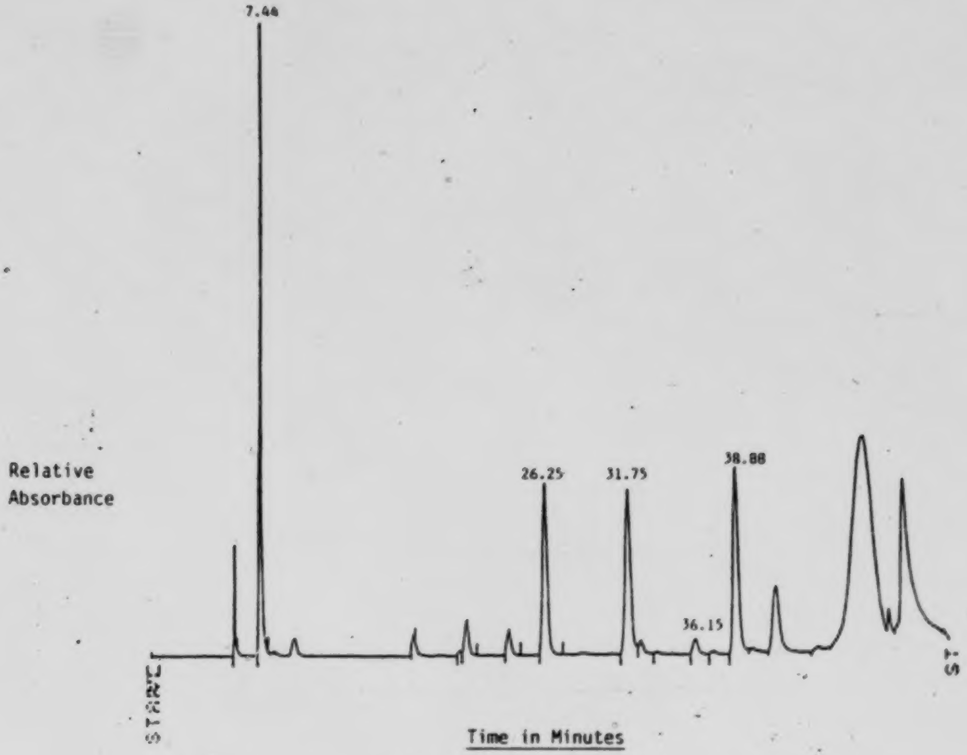
Amino Acid	Time (min.)	Area	Area/Standard	If1=3.40	Whole Number
S	17.24	786	3.40	1.00	1
T	20.21	745	3.67	1.08	1
G	20.65	1078	4.24	1.25	1
R	31.30	932	4.12	1.21	1
L	38.12	1070	4.28	1.26	1

Figure 73. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



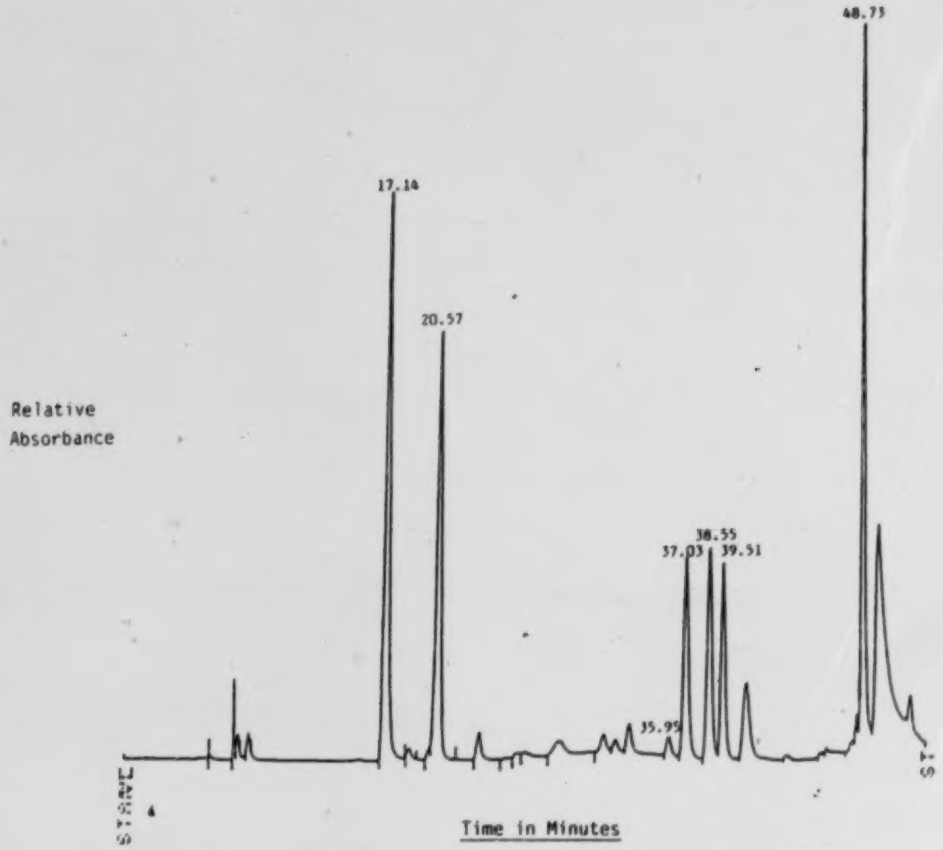
Amino Acid	Time (min.)	Area	Area/Standard	If1=6.03	Whole Number
E	9.56	1150	6.32	1.05	1
G	20.76	2584	10.17	1.69	2
A	23.51	2621	11.20	1.86	2
R	31.11	1388	6.14	1.02	1
V	31.86	1574	6.03	1.00	1
PC	35.63	329	16.12	2.67	3
L	38.37	1519	6.08	1.01	1
K	48.82	1432	5.57	1.09	1

Figure 74. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=4.01	Whole Number
D	7.44	1507	8.92	2.22	2
Y	26.25	991	4.01	1.00	1
R	31.75	1127	4.99	1.24	1
PC	36.15	112	5.49	1.37	1
L	38.00	1181	4.72	1.17	1

Figure 75. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 7 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=4.84	Whole Number
S	17.14	3077	13.32	2.75	3
G	20.57	2377	9.36	1.93	2
PC	35.95	248	12.16	2.51	2
I	37.03	1322	4.93	1.02	1
L	38.55	1288	5.15	1.06	1
F	39.51	1206	4.84	1.00	1
K	48.73	2954	13.55	2.79	3

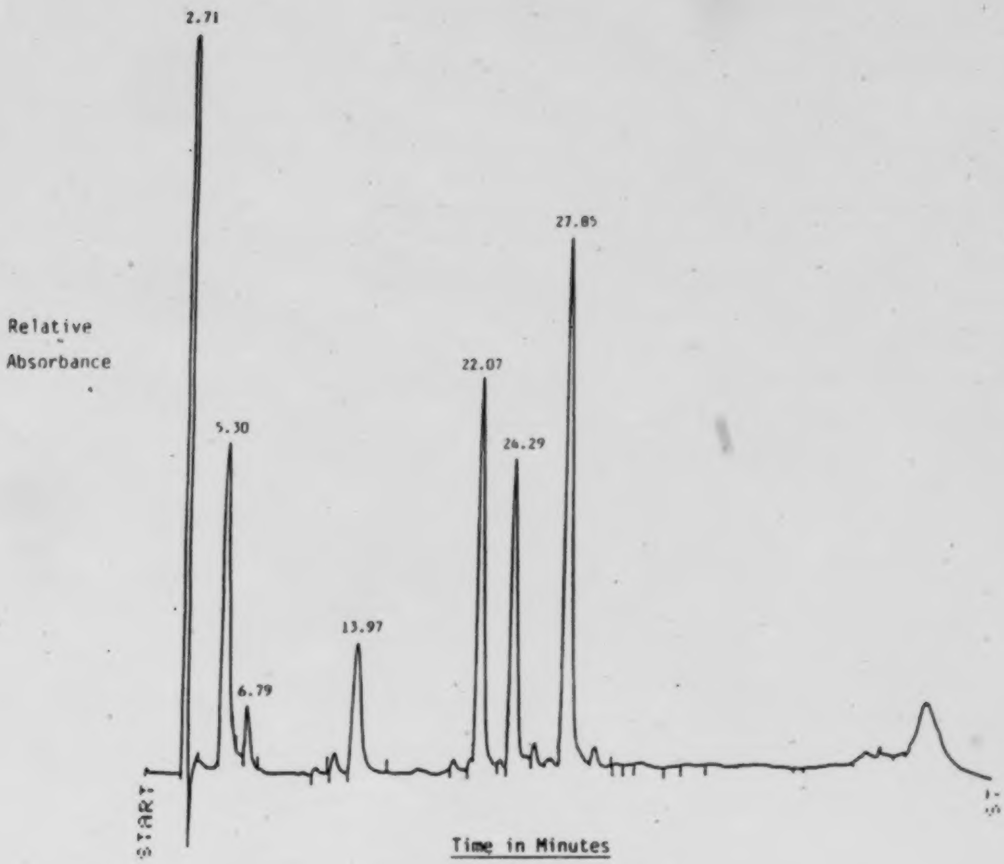
Figure 76. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 8 that resulted from clostripain digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.

Analysis of Purothionin From *T. urartu* and *A. sharonensis*

A number of attempts were made to isolate purothionin from *T. urartu* and *A. sharonensis*. Unfortunately, the equipment available for use was not sensitive enough for the extraction procedure. Seed material was then sent to the USDA facility at Madison Wisconsin where the purothionin was extracted and analysed.

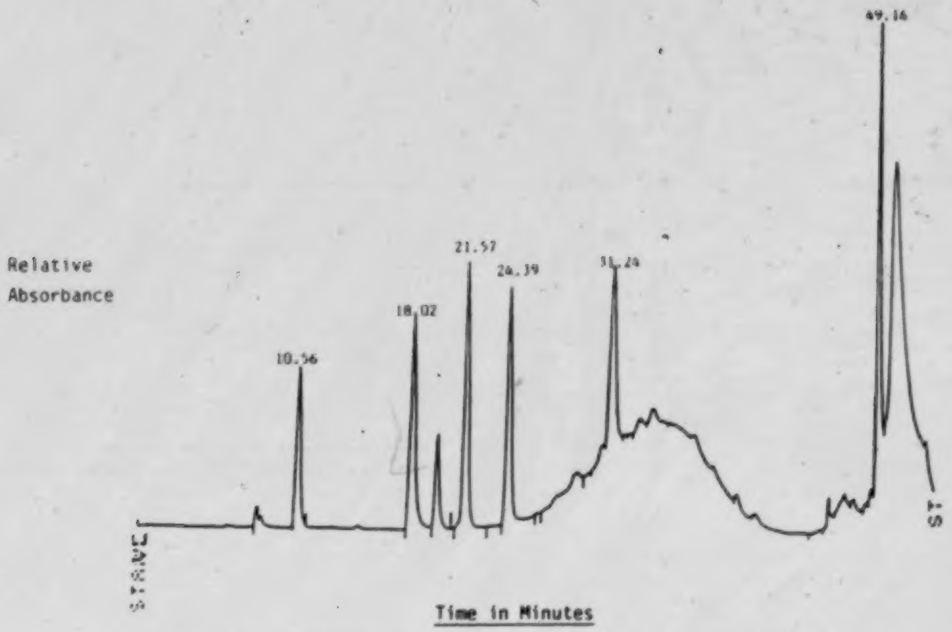
The amino acid sequence of purothionin from *T. urartu* is given in Figure 90. It has an amino acid sequnece identical to β-purothionin.

There were two forms of purothionin isolated from *A. sharonensis* (Figure 91). Each form is different from α_1 purothionin by a single amino acid substitution.



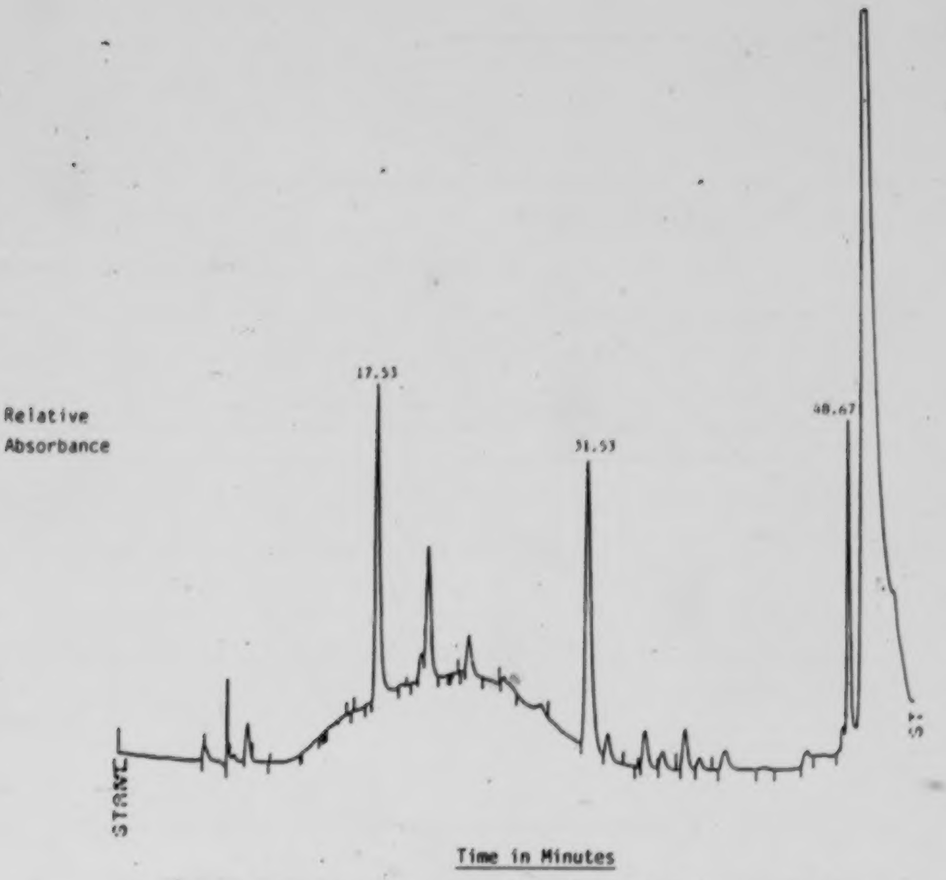
Fraction	Time (min.)
1	2.71
2	5.30
3	6.79
4	13.97
5	22.07
6	24.29
7	27.85

Figure 77. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. searsii* that had been treated with trypsin for 1 hour. Fraction number and elution time is given above.



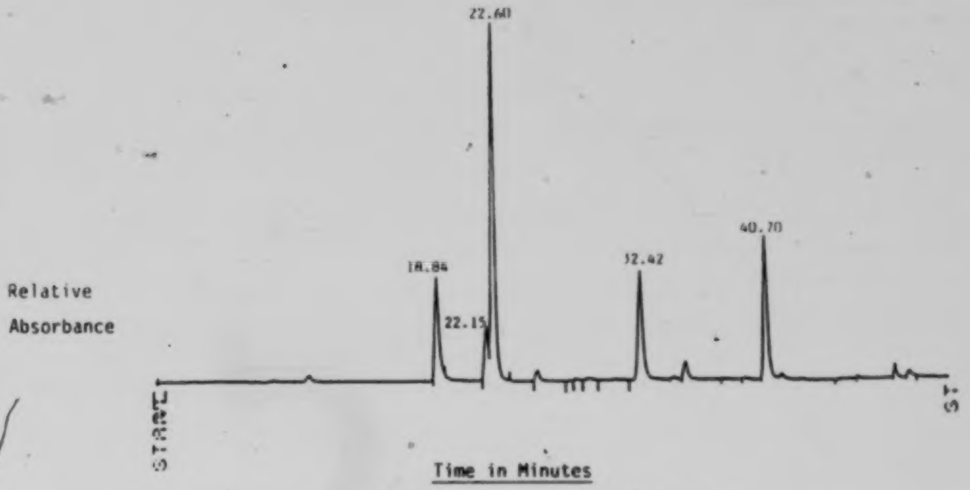
Amino Acid	Time (min.)	Area	Area/Standard	If1=4.57	Whole Number
E	10.56	773	4.57	1.00	1
S	18.02	1301	5.63	1.23	1
G	21.57	1514	5.96	1.30	1
A	24.39	1526	6.52	1.43	1
R	31.24	2784	12.32	2.69	3
K	49.16	2154	9.88	2.16	2

Figure 78. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 1 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



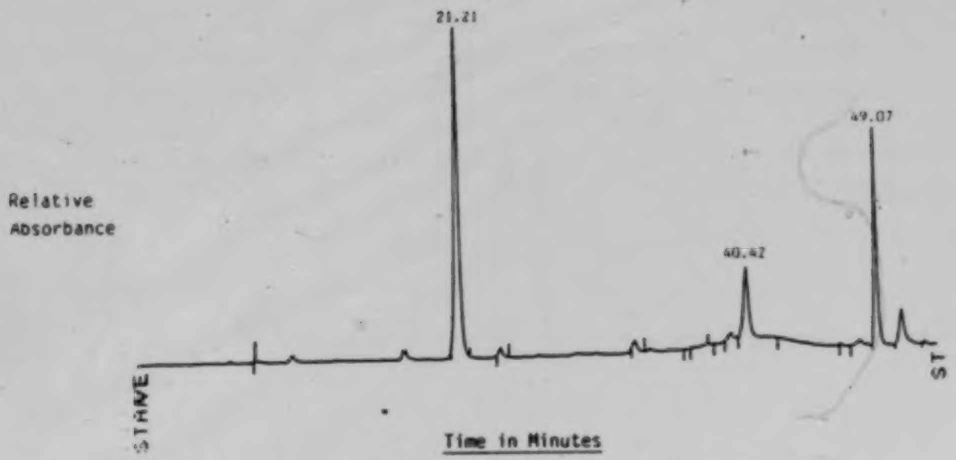
Amino Acid	Time (min.)	Area	Area/Standard	If1=6.05	Whole Number
S	17.53	1787	7.74	1.28	1
R	31.53	2032	8.99	1.49	1
C	37.89	219	10.74	1.78	2
K	48.67	1319	6.05	1.00	1

Figure 79. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 2 that resulted from trypsin digestion from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



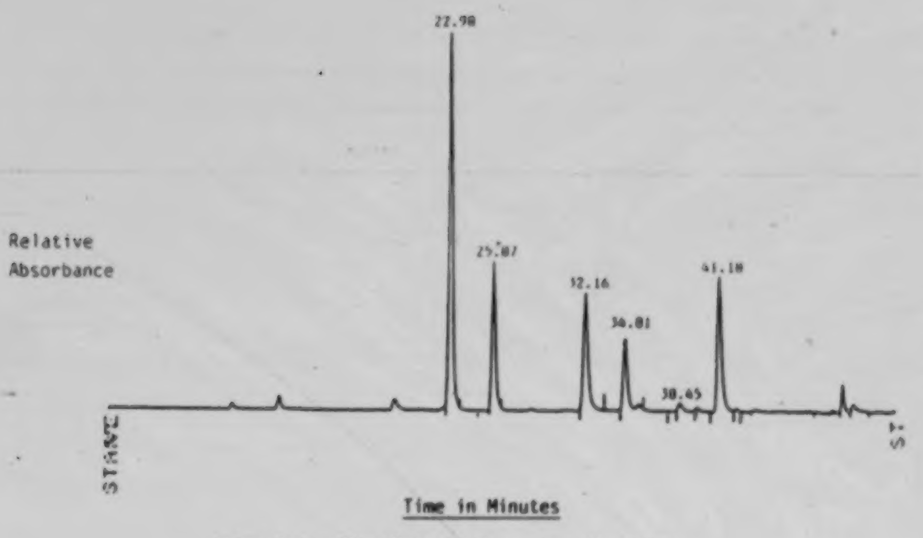
Amino Acid	Time (min.)	Area	Area/Standard
S	18.84	486	2.10
T	22.15	231	1.14
G	22.60	1677	6.60
R	32.42	726	3.21
L	40.70	756	3.02

Figure 80. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 3 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



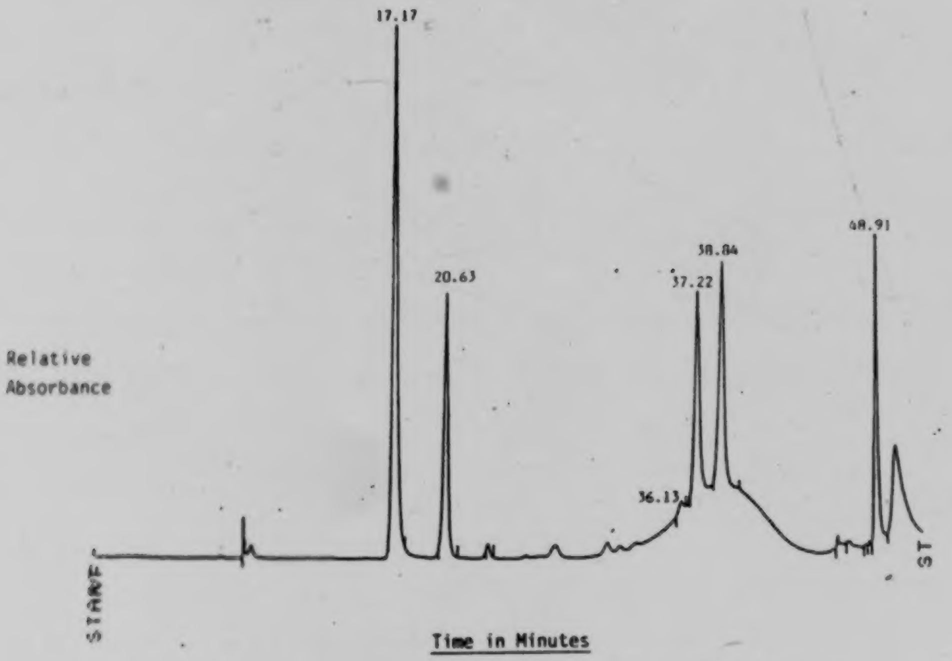
Amino Acid	Time (min.)	Area	Area/Standard
G	21.21	1756	6.91
F	40.42	756	3.04
K	49.07	793	3.64

Figure 81. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 4 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard
G	22.90	1656	6.52
A	25.87	651	2.78
R	32.16	674	2.98
V	34.81	397	1.52
PC	38.45	-	-
L	41.18	715	2.89

Figure 82. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 5 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard	If1=5.13	Whole Number
S	17.17	2857	12.37	2.41	3
G	20.63	1304	5.13	1.00	1
PC	36.13	40	1.96	-	1
I	37.22	1425	5.32	1.04	1
L	38.84	1615	6.46	1.26	1
K	48.91	1231	5.65	1.10	1

Figure 83. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.

EPLC Hydrolysis

The HPLC elution profile of purothionin cleaved with clostripain is given in Figure-85. Six fractions were recovered.

Amino Acid Analysis

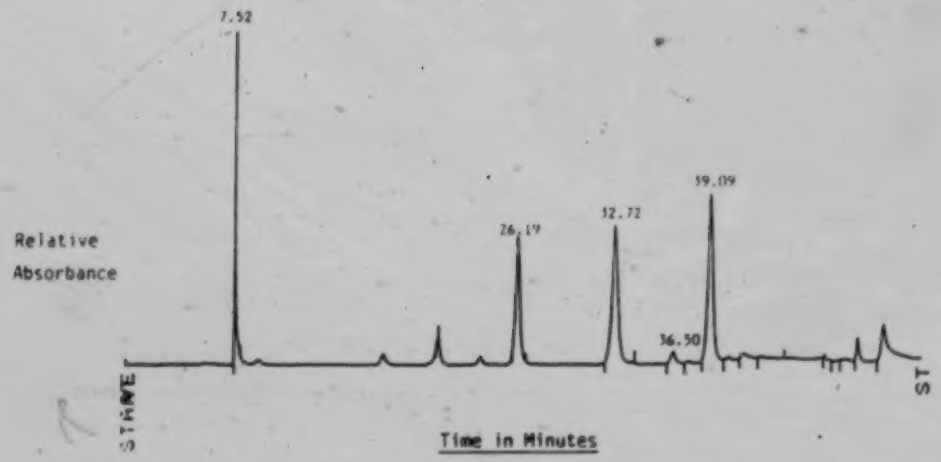
Unfortunately, the OPA used in the analysis of EPLC generated peptides was too old, so the relative amounts of each amino acid could not be determined. However, by comparing the amino acids present in each peptide to that obtained by trypsin and clostripain hydrolysis, the position that each peptide occupies in the protein could be identified.

Fraction 2 contained glycine, phenylalanine, and lysine (Figure 86) and corresponded to amino acids 42 to 45.

Fraction 4 contained serine, glycine, alanine, arginine, valine, isoleucine, leucine, and lysine (Figure 87) and corresponded to amino acids 24-31. The PC peak was not recorded because of the rise in the baseline shortly before the isoleucine peak. Apparently, the lysine at position 32 was not cleaved.

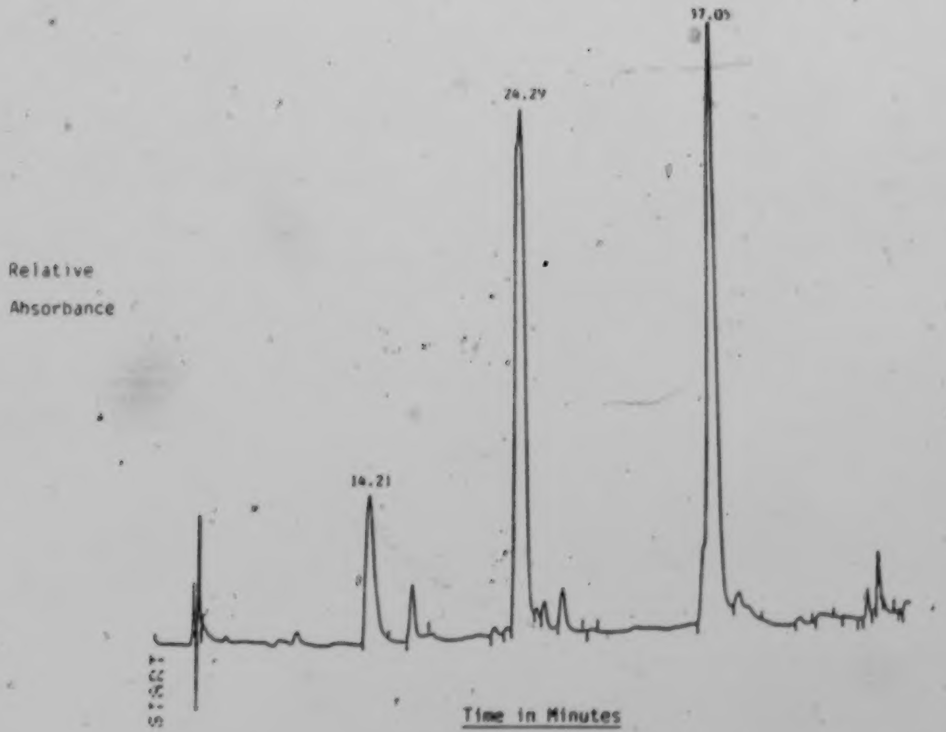
Fraction 6 contained aspartic acid, glutamic acid, serine, threonine, glycine, alanine, tyrosine, arginine, PC, leucine, and lysine (Figure 88) and corresponded to amino acids 2-23.

Based on the above data, the sequence of the purothionin from *A. searsii* is given in Figure 89. The only difference between this sequence and the amino acid sequence of α_1 purothionin is an alanine to serine substitution at position 18.



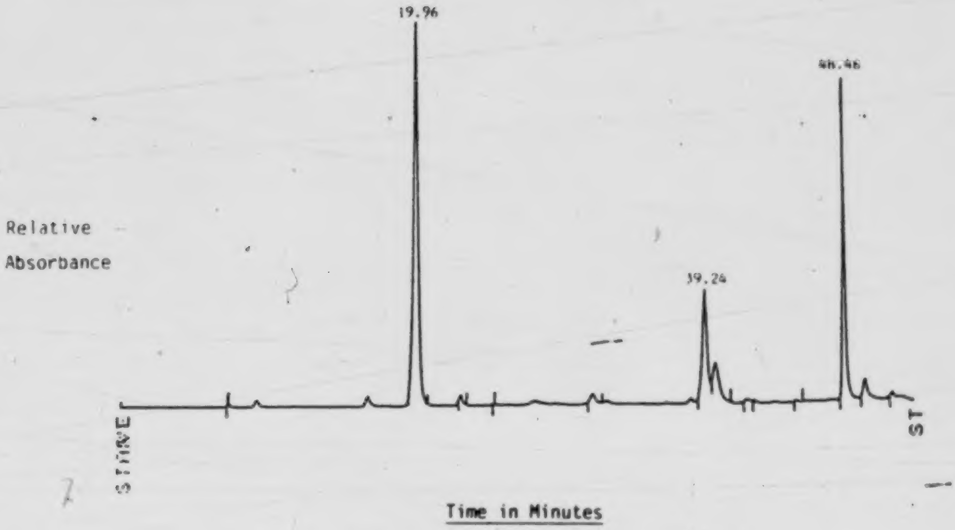
Amino Acid	Time (min.)	Area	Area/Standard
D	7.52	458	2.87
Y	26.19	600	2.43
R	32.72	959	4.24
PC	36.50	-	-
L	39.09	1072	4.29

Figure 84. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 7 that resulted from trypsin digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



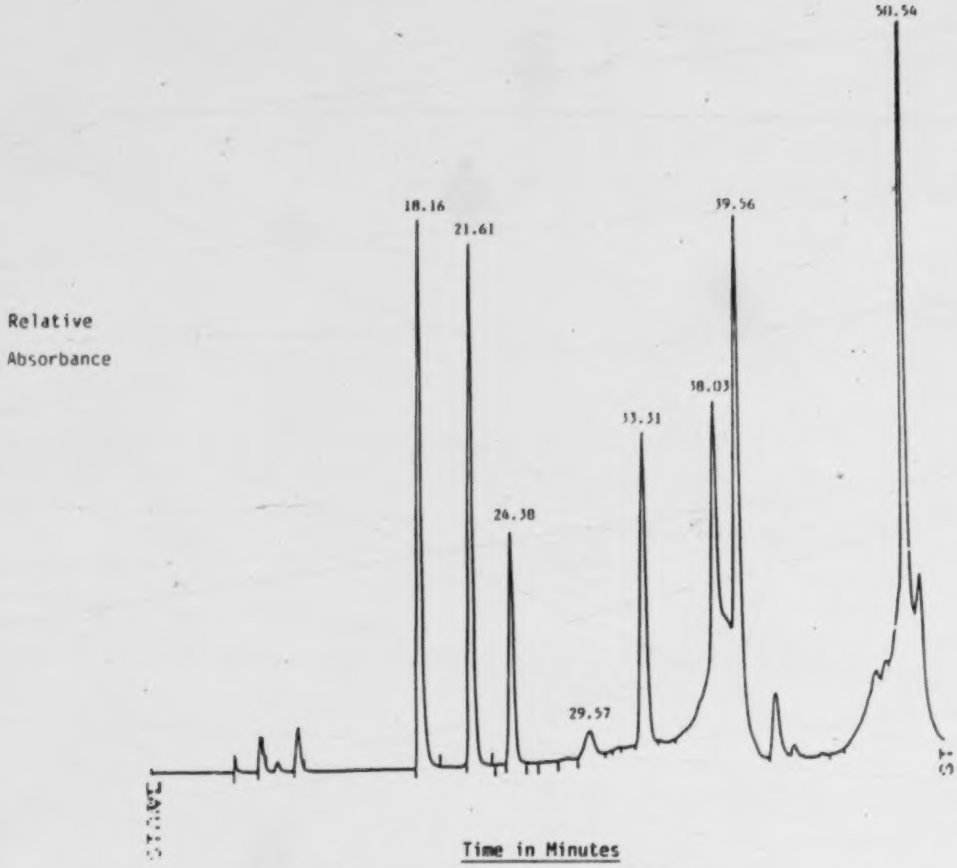
Fraction	Time (min.)
2	14.21
4	24.29
6	37.05

Figure 85. Results from the HPLC separation of peptides formed by the hydrolysis of pyridylethylated purothionin from *A. searsii* that had been treated with EPLC for 1 hour. Fraction number and elution time is given above.



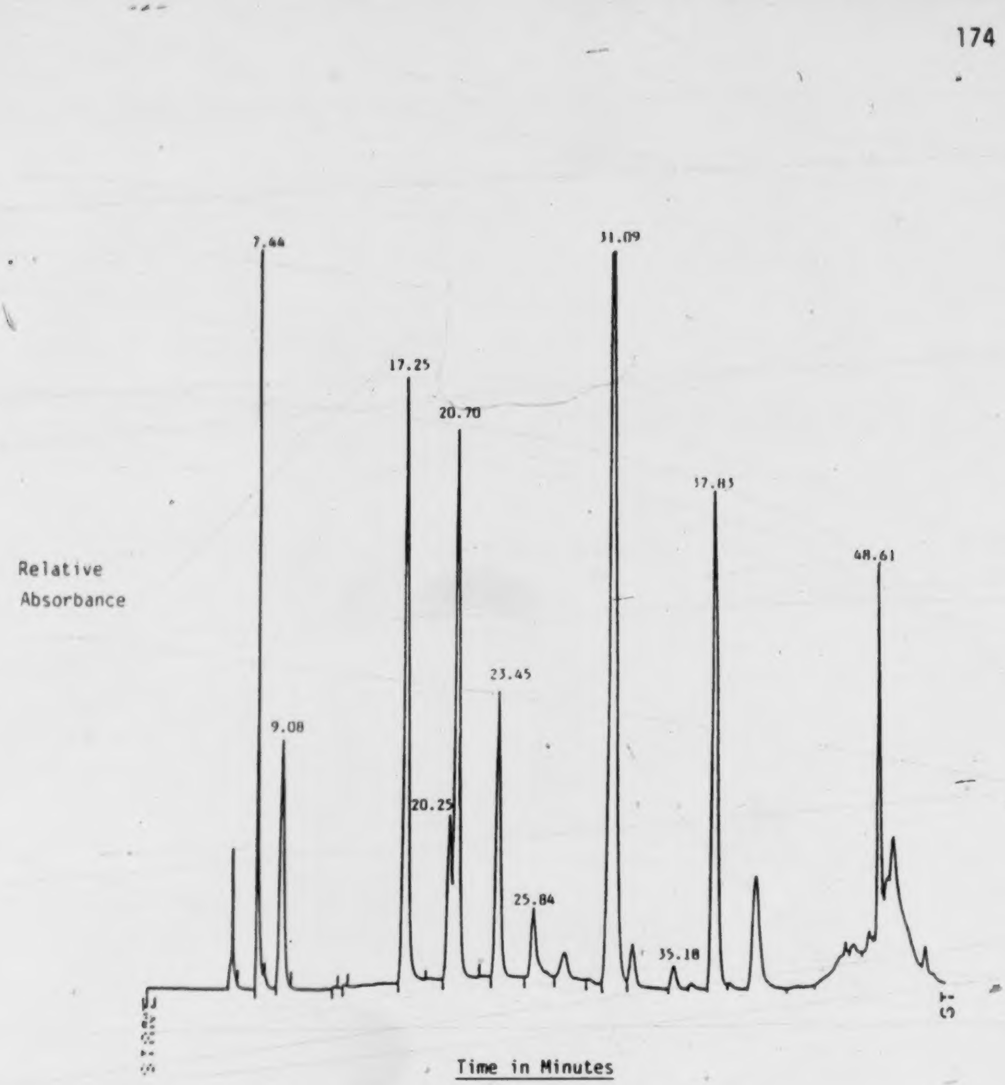
Amino Acid	Time (min.)	Area	Area/Standard
G	19.96	2115	8.33
F	39.24	642	2.58
K	48.46	1136	5.21

Figure 86. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 2 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard
S	18.16	2988	12.94
G	21.61	2863	11.27
A	24.38	1359	5.81
R	29.57	494	2.19
V	33.31	2924	11.20
I	38.03	6584	24.57
L	39.56	4660	18.64
K	50.54	7020	32.20

Figure 87. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 4 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. squarrosa*. Calculations of the number of amino acid residues per peptide is given above.



Amino Acid	Time (min.)	Area	Area/Standard
D	7.44	1586	9.39
E	9.08	1274	7.00
S	17.25	3316	14.36
T	20.25	786	3.87
G	20.70	3145	12.38
A	23.45	1654	7.03
Y	25.84	611	2.47
R	31.09	6496	28.62
PC	35.18	151	7.40
L	37.83	3195	12.78
K	48.61	1855	8.51

Figure 88. Results from the HPLC separation of amino acids formed by HCl hydrolysis of fraction 6 that resulted from EPLC digestion of pyridylethylated purothionin extracted from *A. searsii*. Calculations of the number of amino acid residues per peptide is given above.

K
S
C
C
5 R
S
T
L
G
10 R
N
C
Y
N
15 L
C
R
S
R
20 G
A
Q
K
L
25 C
A
G
V
C
30 R
C
K
I
S
35 S
G
L
S
C
40 P
K
G
F
P
45 K

Figure 89. The amino acid sequence of purothionin isolated from *A. searsii*.

K
S
C
C
5 K
S
T
L
G
10 R
N
C
Y
N
15 L
C
R
A
R
20 G
A
Q
K
L
25 C
A
N
V
C
30 R
C
K
L
T
35 S
G
L
S
C
40 P
K
D
F
P
45 K

Figure 90. The amino acid sequence of purothionin extracted from *T. urartu*.

	K		K	
	S		S	
	C		C	
	C		C	
5	R	5	R	
	S		S	
	T		T	
	L		L	
	G		G	
10	R	10	R	
	N		N	
	C		C	
	Y		Y	
	N		N	
15	L	15	L	
	C		C	
	R		R	
	A		A	
	R		R	
20	G	20	G	
	A		A	
	Q		Q	
	K		K	
	L		L	
25	C	25	C	
	A		A	
	G		G	
	V		V	
	C		C	
30	R	30	R	
	C		C	
	K		K	
	I		I	
	S		S	*
35	S	35	S	*
	G		G	
	V		L	
	S		S	*
	C		C	
40	P	40	P	
	K		K	
	G		G	
	F		F	
	P		P	
45	K	45	K	

Figure 91. The amino acid sequences of the two forms of purotionin extracted from *A. sharonensis*. *The alanine substitution for serine could occur at positions 34, 35, or 38.

Cytological Analysis

The description of the karyotypes of each species is based on centromere position, arm ratio, and the presence or absence of chromosomes with satellites. Except for T. turgidum, in which 8 somatic cell compliments were analyzed, the karyotypes from 10 somatic cells of the other 7 species were studied. Without exception, karyotype variation was observed within each species. For each species, the most common karyotype is described.

The karyotypes of T. monococcum are given in plate 3. A description of each karyotype is given in Table 14. The most common karyotype consisted of 1 St pair, 4 SM pairs, and 2 M pairs of chromosomes.

The karyotypes of T. turgidum var. Stewart are given in plate 4. A description of each karyotype is given in Table 15. The most common karyotype consisted of 2 Sat pairs, 2 St, 7 SM pairs, and 3 M of chromosomes.

The karyotypes of A. speltoides are given in plate 5. A description of each karyotype is given in Table 16. The most common karyotype consisted of 2 Sat pairs, 3 SM pairs, and 2 M pairs of chromosomes.

The karyotypes of A. longissima are given in plate 6. A description of each karyotype is given in Table 17. The most common karyotype consisted of 2 Sat pairs, 1 St pair, and 4 SM pairs of chromosomes.

The karyotypes of A. bicornis are given in plate 7. A description of each karyotype is given in Table 18. The most common karyotype consisted of 1 St pair, 4 SM pairs, and 2 M pairs of chromosomes.

Plate 3. The somatic karyotypes of 10 root-tip cells from *T. monococcum*. Magnification x 1500.

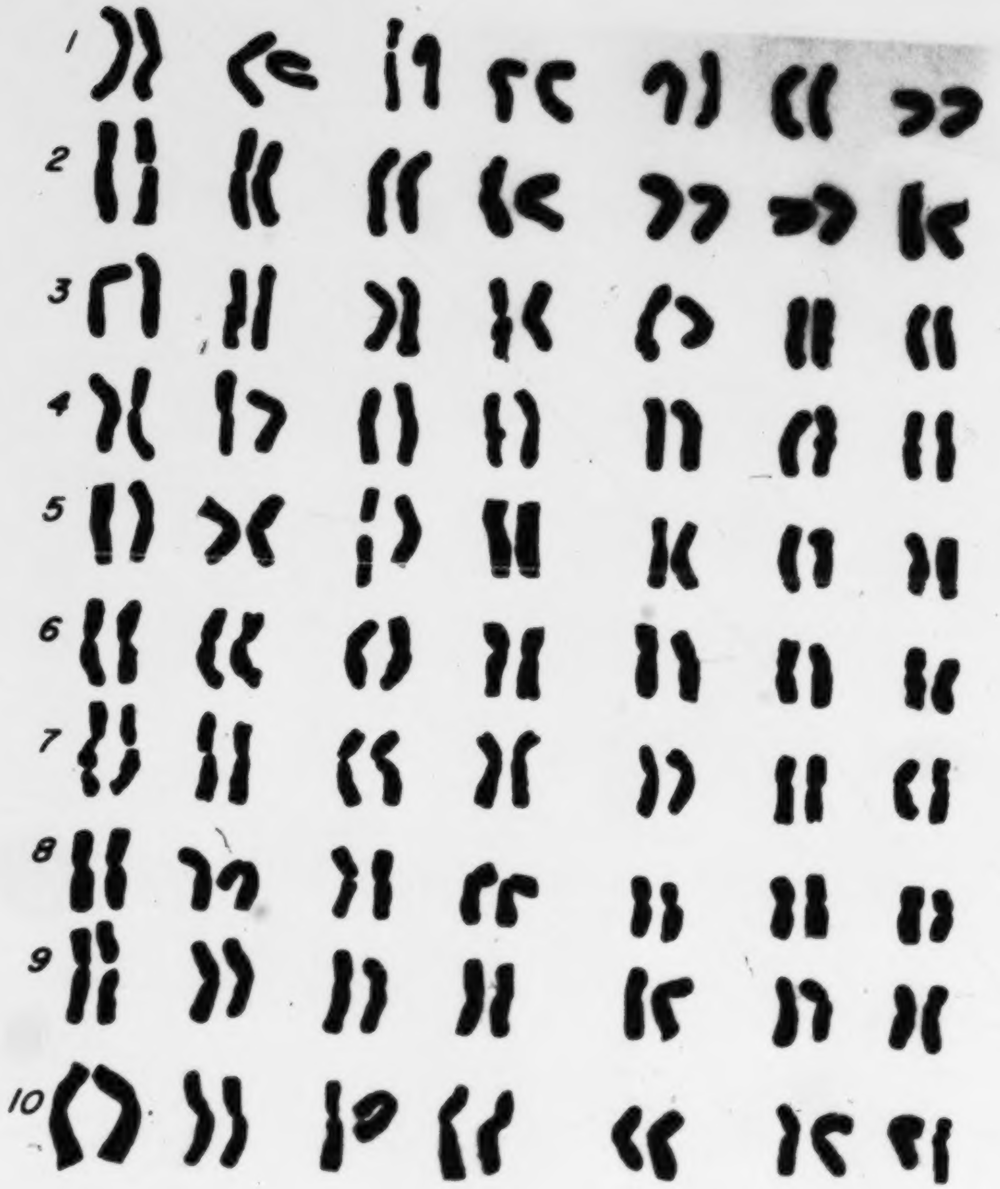
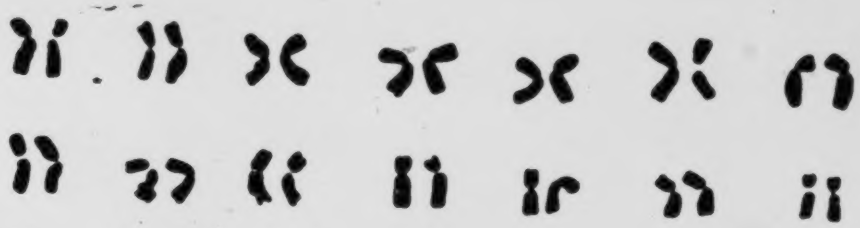
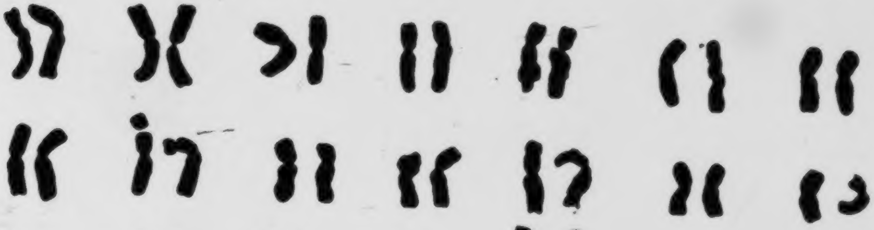


Plate 4. The somatic karyotypes of 4 root-tip cells from *T. turgidum* var. Stewart. Magnification x 1500.

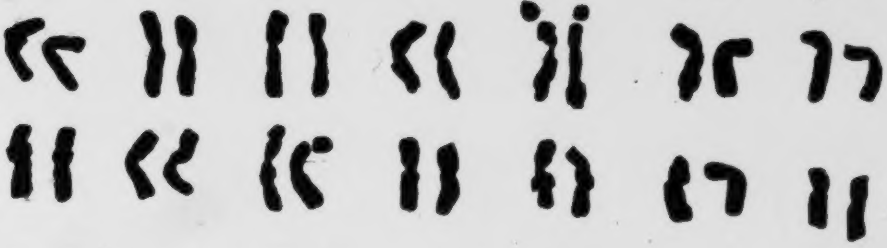
1



2



3



4

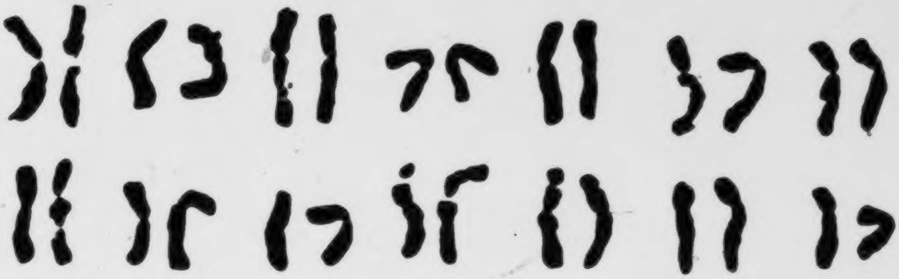
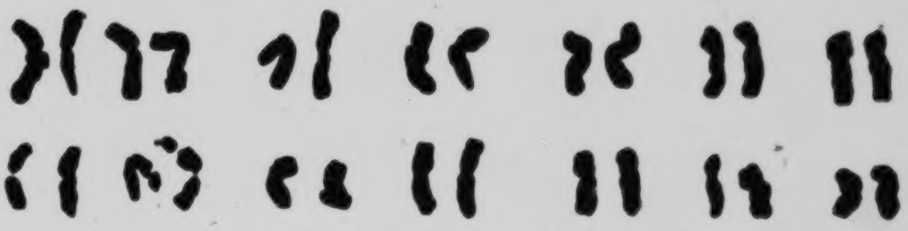
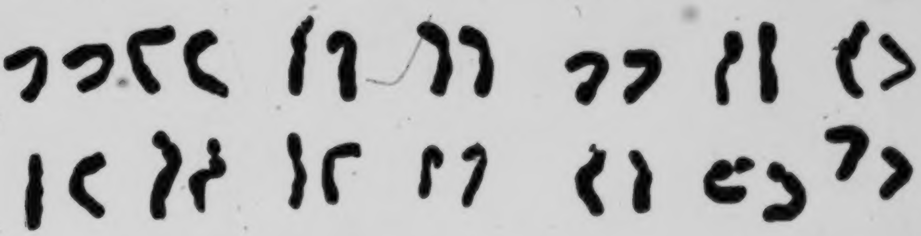


Plate 4 (continued). The somatic karyotypes of 4 root-tip cells from *T. turgidum* var. Stewart. Magnification x 1500.

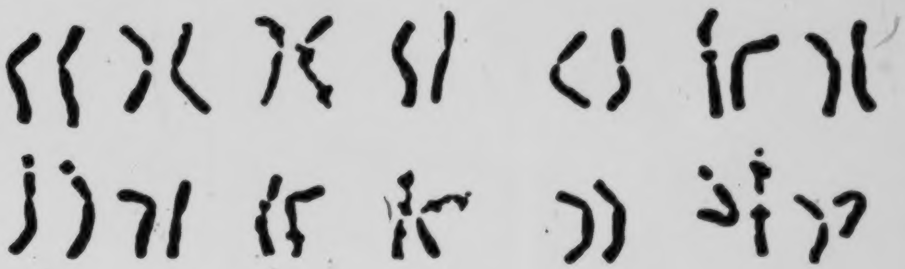
5



6



7



8

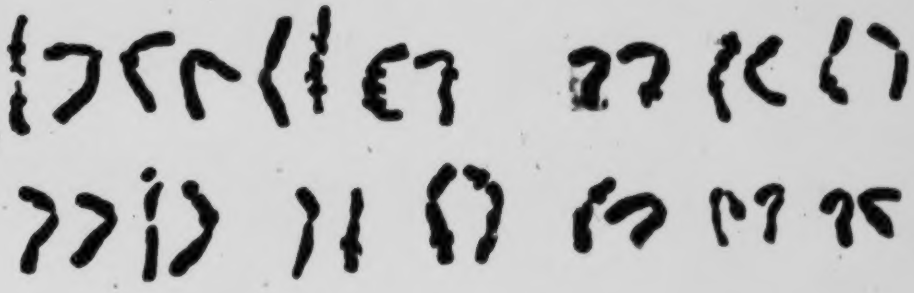


Plate 5. The somatic karyotypes of 10 root-tip cells from *A. speltoides*. Magnification x 1500.

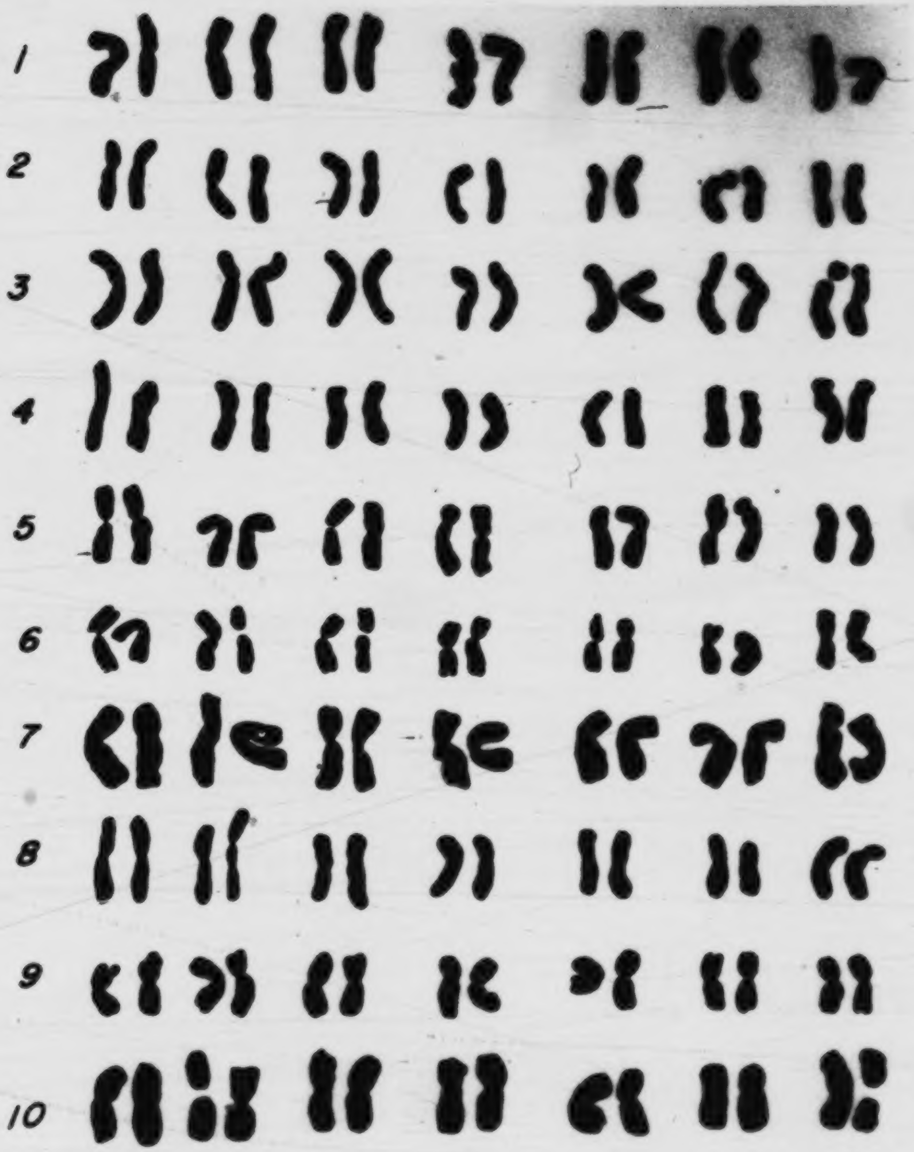


Plate 6. The somatic karyotypes of 10 root-tip cells from *A. longissima*. Magnification x 1500.

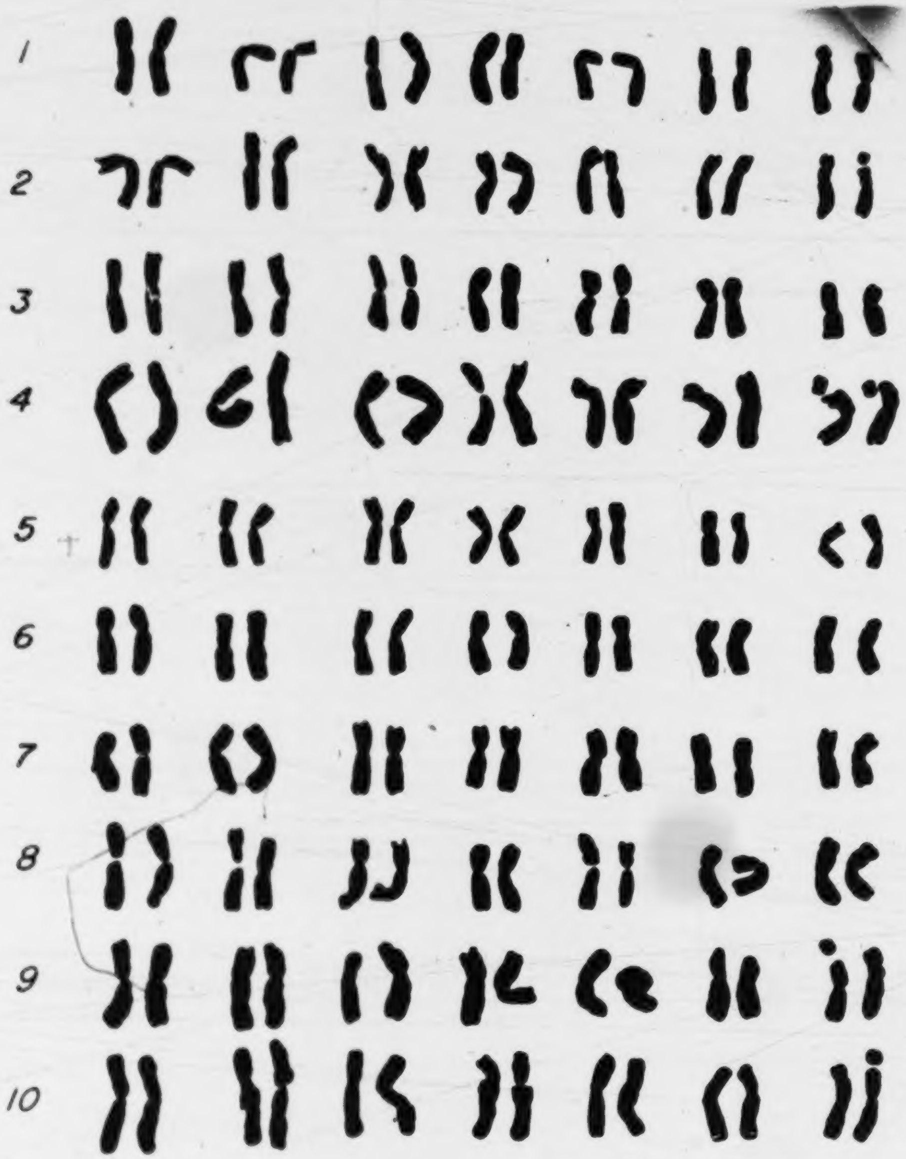
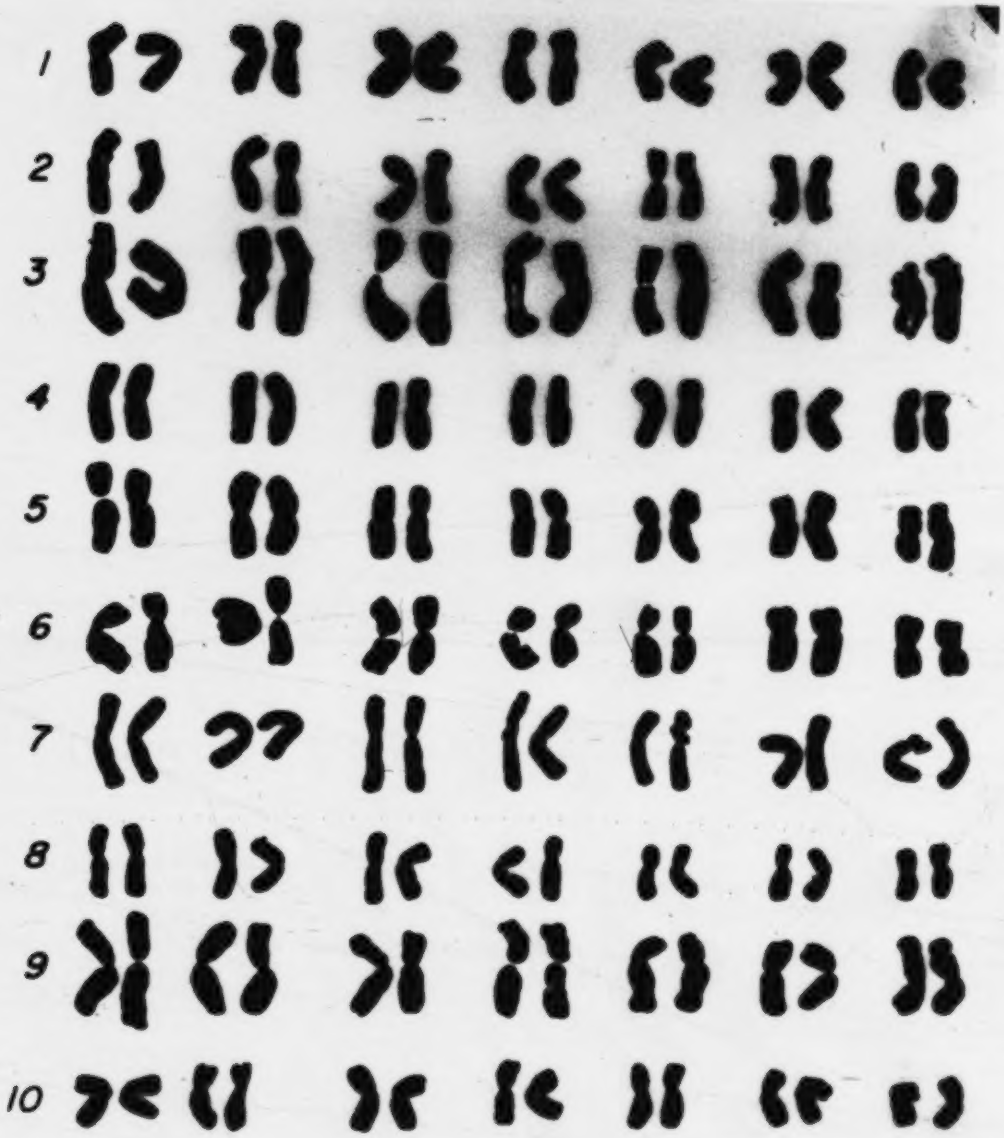


Plate 7. The somatic karyotypes of 10 root-tip cells from *A. bicornis*. Magnification x 1500.



1
2
3
4
5
6
7
8
9
10

Plate 8. The somatic karyotype of 10 root-tip cells from *A. searsii*. Magnification x 1500.

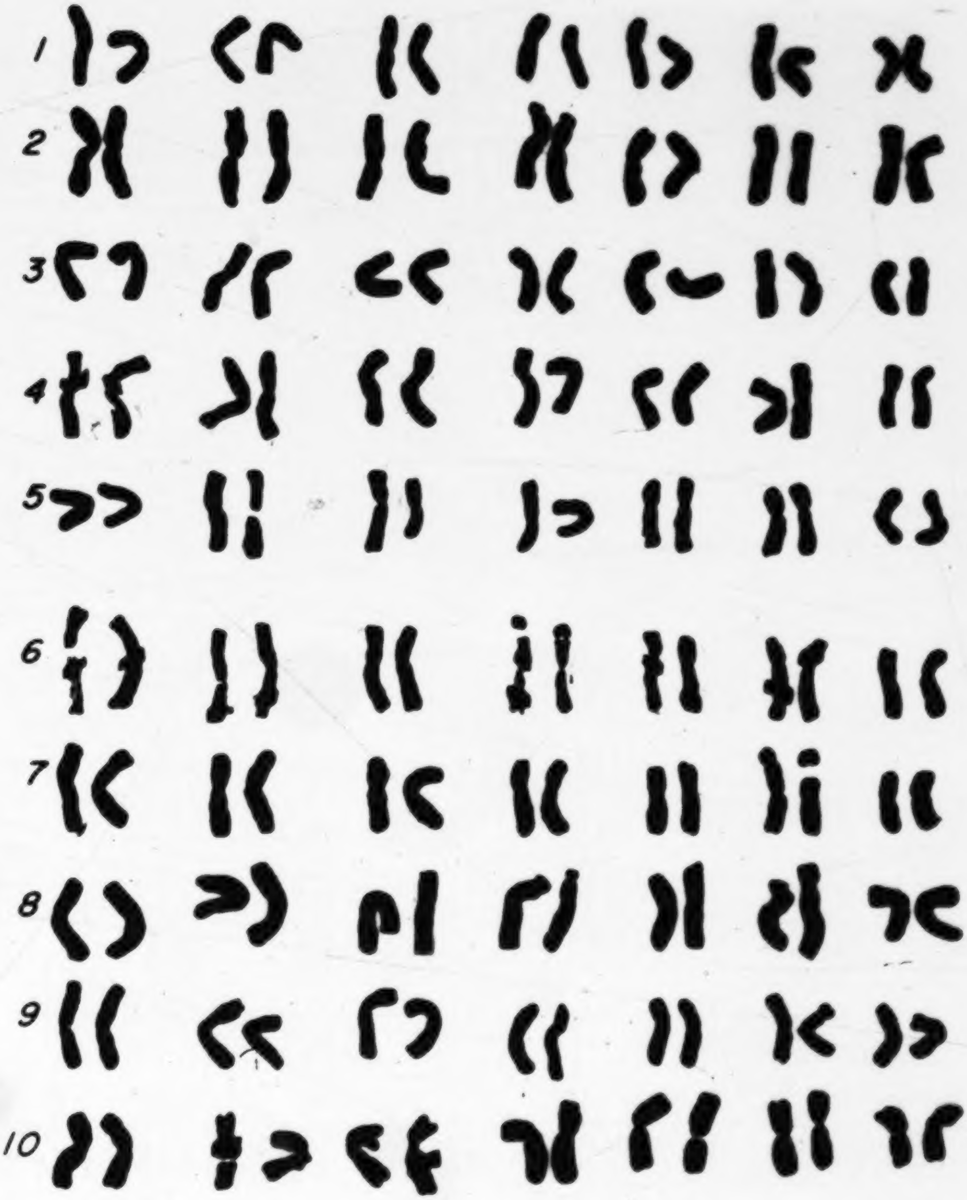


Plate 9. The somatic karyotype of 10 root-tip cells from *A. sharonensis*. Magnification x 1500.

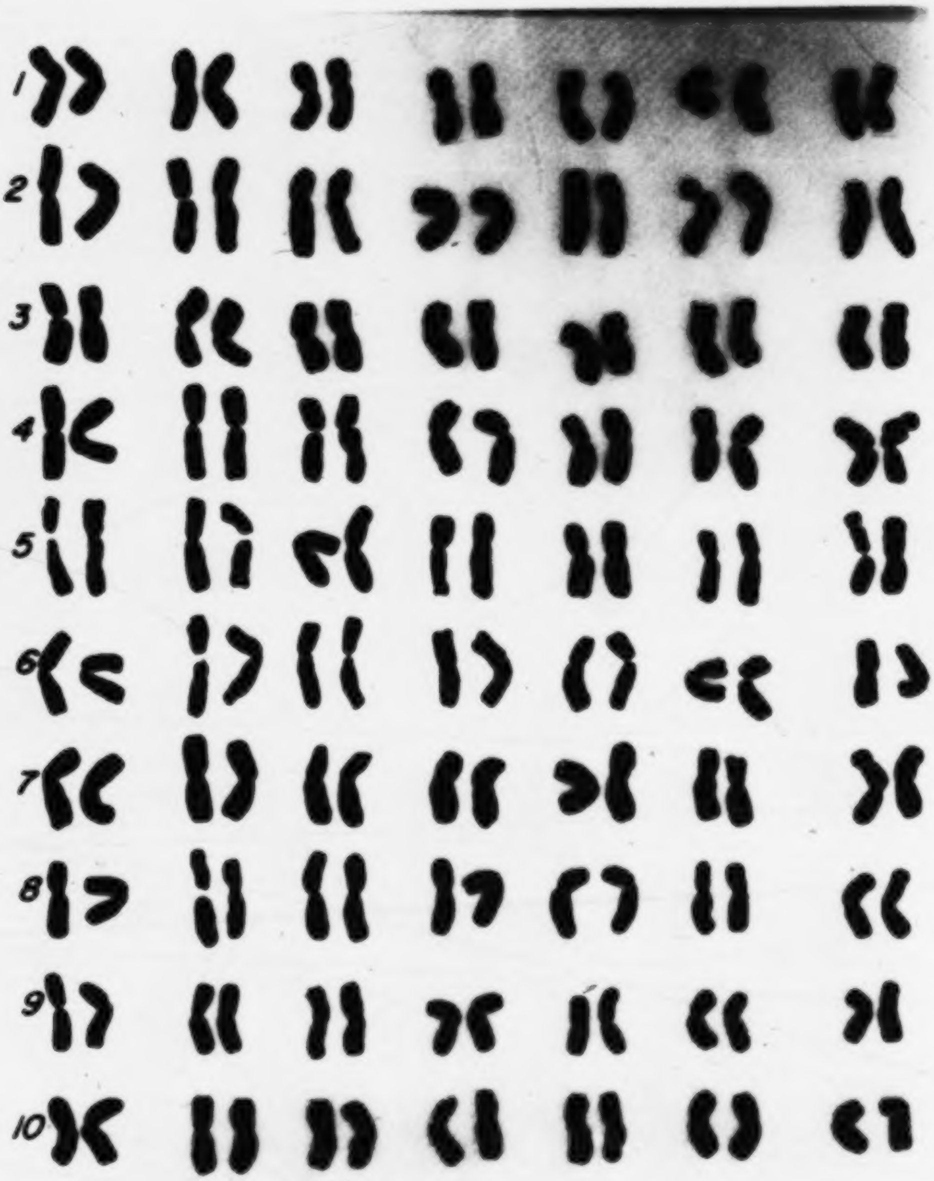
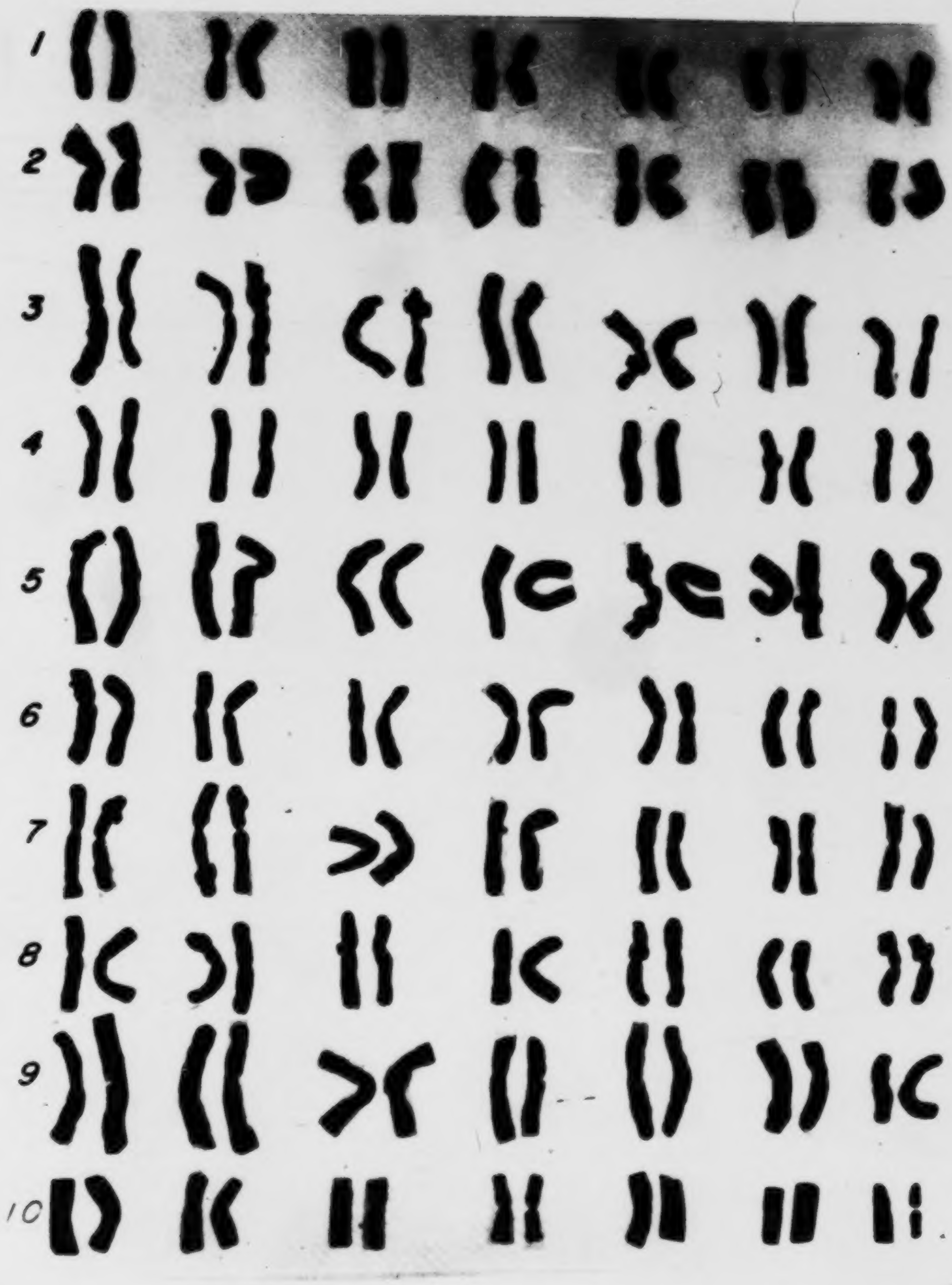

1
2
3
4
5
6
7
8
9
10

Plate 10. The somatic karyotype of 10 root-tip cells from *T. urartu*. Magnification x 1500.


1
2
3
4
5
6
7
8
9
10

The karyotypes of _A_. _searsii_ are given in plate 8. A description of each karyotype is given in Table 19. The most common karyotype consisted of 2 Sat pairs, 1 St pair, 3 SM pairs, and 1 M pair of chromosomes.

The karyotypes of _A_. _sharonensis_ are given in plate 9. A description of each karyotype is given in Table 20. The most common karyotype consisted of 1 Sat pair, 1 St pair, 4 SM pairs, and 1 M pair of chromosomes.

The karyotypes of _T_. _urartu_ are given in plate 10. A description of each karyotype is given in Table 21. The most common karyotype consisted of 2 St pair, 3 SM pairs, and 2 M pairs of chromosomes.

Karyotype Number	Sat	St	SM	M
1	0	2	3	2
2	0	2	4	1
3	0	1	4	2
4	0	1	4	2
5	0	2	3	2
6	0	0	5	2
7	0	2	4	1
8	0	1	5	1
9	0	2	2	3
10	0	1	3	3

Table 14. The description of each karyotype from _T_. _monococcum_.

Karyotype Number	Sat	St	SM	M
1	0	4	7	3
2	2	3	7	2
3	2	4	7	1
4	2	2	7	3
5	2	7	4	1
6	2	5	5	2
7	2	3	5	4
8	2	3	5	4

Table 15. The description of each karyotype from _T_. _turgidum_.

Karyotype Number	Sat	St	SM	M
1	2	0	3	2
2	2	0	2	3
3	2	0	2	3
4	1	0	2	4
5	1	1	3	2
6	1	0	4	2
7	1	0	4	2
8	0	1	3	3
9	0	1	3	3
10	1	0	3	3

Table 16. The description of each karyotype from *A. speltoides*.

Karyotype Number	Sat	St	SM	M
1	1	1	5	0
2	1	2	4	0
3	0	1	4	2
4	2	1	2	2
5	0	2	4	1
6	1	1	4	1
7	0	0	6	1
8	2	1	4	0
9	2	0	4	1
10	2	1	4	0

Table 17. The description of each karyotype from *A. longissima*.

Karyotype Number	Sat	St	SM	M
1	0	2	2	3
2	0	1	4	2
3	1	0	4	2
4	0	2	1	4
5	1	0	5	1
6	0	1	3	3
7	2	1	2	2
8	0	1	3	3
9	1	1	3	2
10	0	1	4	2

Table 18. The description of each karyotype from _A_. _bicornis_.

Karyotype Number	Sat	St	SM	M
1	2	1	1	3
2	2	0	3	2
3	1	2	2	2
4	2	1	3	1
5	2	1	2	2
6	2	1	3	1
7	1	0	6	0
8	2	0	4	1
9	1	2	1	3
10	1	0	5	1

Table 19. The description of each karyotype from _A_. _searsii_.

Karyotype Number	Sat	St	SM	M
1	1	1	4	1
2	2	1	3	1
3	2	1	2	2
4	1	2	3	1
5	1	1	4	1
6	1	2	2	2
7	1	0	5	1
8	1	1	4	1
9	1	0	4	2
10	1	0	4	2

Table 20. The description of each karyotype from *A. sharonensis*.

Karyotype Number	Sat	St	SM	M
1	0	2	3	2
2	0	0	3	4
3	0	0	5	2
4	0	2	3	2
5	1	1	1	4
6	0	1	5	1
7	0	1	3	3
8	1	1	3	2
9	1	0	4	2
10	0	1	3	3

Table 21. The description of each karyotype from *T. urartu*.

DISCUSSION

The quest to identify the B genome donor to *T. turgidum* and *T. aestivum* has gone on for more than 60 years without unequivocally identifying one obvious species. In the case of *A. speltoides* one can locate at least 15 references supporting this species as the donor and another 15 refuting it. These studies include factors from plant morphology to biochemical analysis. This being the case, one may have to rely on complementary studies to identify the specific donor.

It was felt that the determination of the amino acid sequence of the purothionin from each putative B genome donor species would add critical information about the evolutionary relationship of these species. Purothionins are the first example of a completely conserved, genome specific protein that is present in the diploid, tetraploid, and hexaploid *Triticum* and *Aegilops* species.

The cytological analysis was unique since this was the first study that has used a standard procedure that included all putative B genome donors. In addition, this was the first study to employ computer assisted measurements of the chromosome arms from the karyotype of each species.

Discussion of Purothionin Results

The amino acid sequence of α_1, α_2, β-purothionins, and the purothionins from the putative B genome donors is given in Figure 92. The B genome donor to the polyploid wheats should contain a purothionin with an amino acid sequence similar to the α_1-purothionin sequence.

The amino acid sequence of purothionin isolated from A. speltoides differed from α_1-purothionin by 2 amino acids substitutions, from α_2-purothionin by 7 amino acid substitutions, and β-purothionin by 5 amino acid substitutions. On the basis of amino acid sequence homology, A. speltoides is an unlikely candidate as the B genome donor to the polyploid wheats.

The amino acid sequence of purothionin isolated from A. longissima differed from α_1-purothionin by 6 amino acids, from α_2-purothionin by 0 amino acids, and β-purothionin by 7 amino acids. Therefore, on the basis of purothionin sequence, A. longissima is an unlikely candidate as the B genome donor. It was surprising that the purothionin from A. squarrosa and A. longissima had an identical amino acid sequence. These two species are morphologically distinct, have different habitats, and do not overlap in distribution (Witcombe, 1983). One explanation for these results is that seeds from two different sources were used in the protein experiments. Initially there was a problem obtaining A. longissima seeds. Various lines of A. longissima seed obtained from Ottawa and Beltsville had to be discarded because it turned out to be A. caudata. Seed from A. longissima is currently being increased for analysis.

There is 1 amino acid difference in the sequence of

β	α_2	α_1	1	2	3	4	5	6	6a
K	K	K	K	K	K	K	K	K	K
S	S	S	S	S	S	S	S	S	S
C	C	C	C	C	C	C	C	C	C
C	C	C	C	C	C	C	C	C	C
K	R	R	R	R	R	R	K	R	R
S	T	S	S	T	S	S	S	S	S
T	T	T	T	T	t	T	T	T	T
L	L	L	L	L	L	L	L	L	L
G	G	G	G	G	G	G	G	G	G
R	R	R	R	R	R	R	R	R	R
N	N	N	N	N	N	N	N	N	N
C	C	C	C	C	C	C	C	C	C
Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
N	N	N	N	N	N	N	N	N	N
L	L	L	L	L	L	L	L	L	L
C	C	C	C	C	C	C	C	C	C
R	R	R	R	R	R	R	R	R	R
A	S	A	A	S	A	S	A	A	A
R	R	R	R	R	R	R	R	R	R
G	G	G	G	G	G	G	G	G	G
A	A	A	A	A	A	A	A	A	A
Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
K	K	K	K	K	K	K	K	K	K
L	L	L	L	L	L	L	L	L	L
C	C	C	C	C	C	C	C	C	C
A	S	A	A	S	A	A	A	A	A
N	T	G	G	T	G	G	N	G	G
V	V	V	V	V	V	V	V	V	V
C	C	C	C	C	C	C	C	C	C
R	R	R	R	R	R	R	R	R	R
C	C	C	C	C	C	C	C	C	C
K	K	K	K	K	K	K	K	K	K
L	L	I	I	L	I	I	L	I	I
T	T	S	A	T	A	S	T	S	A
S	S	S	S	S	S	S	S	S	S
G	G	G	G	G	G	G	G	G	G
L	L	L	V	L	L	L	L	V	L
S	S	S	S	S	S	S	S	S	S
C	C	C	C	C	C	C	C	C	C
P	P	P	P	P	P	P	P	P	P
K	K	K	K	K	K	K	K	K	K
D	G	G	G	G	G	G	D	G	G
F	F	F	F	F	F	F	F	F	F
P	P	P	P	P	P	P	P	P	P
K	K	K	K	K	K	K	K	K	K

Figure 92. The amino acid sequence of β, α_2, and α_1-purothionin (Jones et al., 1982), and purothionin from: 1-*A. speltoides*, 2-*A. longissima*, 3-*A. bicornis*, 4-*A. searsii*, 5-*T. urartu*, 6-*A. sharonensis*.

α_1-purothionin and purothionin isolated from *A. bicornis*. The difference is a serine to alanine substitution at position 34, 35, or 38. To determine the exact position of the substitution, a peptide containing this region would have to be sequenced. On the basis of purothionin sequence, *A. bicornis* should be considered as a candidate for the B genome donor to the wheats. Purothionin from *A. bicornis* differed from α_2-purothionin by 6 amino acids and β-purothionin by 4 amino acid substitutions.

Purothionin from *A. searsii* differed from the amino acid sequence α_1-purothionin by an alanine to serine substitution at position 18. It differed from α_2-purothionin by 5 amino acids and β-purothionin by 4 amino acids. On the basis of the amino acid sequence of purothionin, *A. searsii* appears to be a possible candidate as the B genome donor to the polyploid wheats.

Two forms of purothionin were isolated from *A. sharonensis*. Together, they differed from α_1-purothionin by 2 amino acids, α_2-purothionin by 7 amino acids, and β-purothionin by 5 amino acids. The differences from α_1-purothionin were identical to the difference between purothionin from *A. speltoides* and α_1-purothionin. It will be interesting to see if *A. speltoides* contains two forms of purothionin used for extracting purothionin from *A. sharonensis*. On the basis of the amino acid sequence of purothionins, *A. sharonensis* is an unlikely candidate as B genome donor to polyploid wheats.

The amino acid sequence of purothionin isolated from *T. urartu* differed from α_1-purothionin by 4 amino acids, α_2-purothionin by 7 amino acids and β-purothionin by 0 amino acids. Therefore, on the basis of the amino acid sequence of purothionin, *T. urartu* cannot be considered as a likely candidate of the B genome donor to the polyploid wheats. It was not unexpected that the sequence of purothionin from _T. urartu_ was identical to β-purothionin. As was pointed out in the literature review, Chapman _et al_. (1976) and Dvořák (1976) found that the A genome chromosomes in _T. aestivum_ paired with the chromosomes from _T. urartu_ during meiosis in hybrids produced by crossing these two species.

Cytological Analysis

This is the first study that has analyzed the karyotypes of all the potential B genome donor species using an identical pretreatment, fix, and straining procedure. It has been difficult to assess the conclusions from previous studies because a variety of methods have been used in tissue preparation. For example, Riley et al. (1958) pretreated with bromonapthalene for 3-4 hours, fixed in glacial acetic acid, hydrolysed in 1N HCl at 60°C for 12 minutes, and stained in basic fuchsin; Giorgi and Bozzini (1969) pretreated with bromonapthalene for 4 hours, fixed in acetic-alcohol (1:3), hydrolysed in 1N HCl at 60°C for 7 minutes, stained with Feulgen, treated with 5% prectinase at 40°C for 20-25 minutes; and Kushnir and Halloran (1981) pretreated with 0.001% colchicine for 24 hours and stained in 1% acetocarmine for 48 hours before squashing.

This study incorporated some of the most advanced equipment available for measuring chromosome arm lengths and determining arm ratio. Previous studies (Giorgi and Bozzini, 1969 a-c, and Kushnir and Halloran, 1981) have not indicated how the length of each chromosome arm was determined.

To determine the description of the karyotype of the B genome donor to the wheats, the karyotypes of T. turgidum and T. monococcum were obtained. Based on the description of the chromosomes from T. monococcum, the A genome chromosomes in the T. turgidum karyotype could be identified, leaving a description of the B genome chromosomes.

Karyotype Analysis of *T. monococcum*

The karyotype of T. monococcum has been reported to contain 1 chromosome pair with small satellites (Riley et al., 1958; Upadhya and Swaminathan, 1963; Coucoli and Skorda, 1966) or 2 chromosome pairs with small satellites (Pathak, 1940; Giorgi and Bozzini, 1969b). Waines and Kimber (1973) found that variation in the size and number of satellites was dependent on the geographical origin of the seed. One or two pairs of chromosomes with satellites were observed.

During this current study, cells containing 2 pairs of chromosomes with satellites were rarely observed and cells containing one chromosome pair with satellites were occasionally observed (plates not shown). The great majority of cells observed did not contain chromosomes with satellites. The most common karyotype observed in this study consisted of 1 St pair, 4 SM pairs, 2 M pairs of chromosomes. Giorgi and Bozzini (1969b) observed 2 Sat pairs, 3 SM pairs, and 2 M pairs of chromosomes in T. monococcum. One of the Sat chromosome pairs had a St arm ratio while the other Sat pair had a SM arm ratio. The results of this present study and that from Giorgi and Bozzini (1969b) are in agreemnet if one assumes that the St pair and one of the SM pairs observed in this study are the same as the 2 Sat pairs of chromosomes in the previous study.

Karyotype Analysis of *T. turgidum*

The karyotype description of T. turgidum is based on the analysis of 8 cells. One of the most common descriptions of T. turgidum is 2 Sat pairs, 2 St pairs, 7 SM pairs, and 3 M pairs of chromosomes. This description was chosen over other equally frequent descriptions because it agreed with the results of Giorgi and Bozzini (1969a) which was based on 130 cells in total and Kushnir and Halloran (1981) which was based on 1 cell.

Variation in karyotype morphology was observed in this species. Occasionally, a cell was observed that contained all the chromosomes without satellites (plate line 1). This observation may be a result of the level of chromosome coiling since the chromosomes in this cell are relatively smaller than any others observed in this study. Other cells from the same root contained chromosomes with satellites so variation in chromosome morphology can occur between cells within the same tissue.

Karyotype of the B Genome

Since the karyotype of *T. turgidum* consisted of 2 Sat pairs, 2 St pairs, 7 SM pairs, and 3 M pairs of chromosomes, and *T. monococcum* consisted of 1 St pair, 4 SM pairs, and 2 M pairs of chromosomes, the karyotype of the B genome donor should consist of 2 Sat pairs, 1 St pair, 3 SM pairs, and 1 M pair of chromosomes.

Karyotype Analysis of the Putative B Genome Donors to Tetraploid and Hexaploid Wheats

Pathak (1940) reported that the karyotype of *A. speltoides* consisted of 2 Sat pairs, 4 SM pairs, and 1 M pair of chromosomes. Riley et al. (1958) confirmed the observation that 2 pairs of chromosomes with large satellites occur in *A. speltoides* and proposed the *A. speltoides* was the B genome donor to tetraploid and hexaploid wheats. Giorgi and Bozzini (1969b) reported that the karyotype of *A. speltoides* consisted of 2 Sat pairs, 1 St pair, 2 SM pairs, and 2 M pairs of chromosomes.

The most common karyotype observed in this study consisted of 2 Sat pairs, 3 SM pairs, and 2 M pairs of chromosomes. Since the B genome donor is expected to have 2 Sat pairs, 1 St pair, 3 SM pairs, and 1 M pair of chromosomes, *A. spletoides* can be excluded as the donor of the B genome on this basis.

Senyaninova-Korchagina was the first to report that the karyotype of *A. longissima* contained two pairs of Sat chromosomes (referenced by Chennaveeraiah, 1960). This observation was confirmed by Riley et al. (1958). They observed one pair with large and one pair with small satellites. According to Chennaveeraiah (1960) all other chromosome pairs are sub-median.

The most common karyotype observed in this study consisted of 2 Sat pairs, 1 St pair, and 4 SM pairs of chromosomes. One pair of chromosomes contains large satellite and another pair contains small satellites. On the basis of karyotype morphology, *A. longissima* cannot be the B genome donor.

Senyaninova-Korchagina (referenced by Chennaveeraiah, 1960) was the first to report that the karyotype of *A. bicornis* contained one pair of chromosomes with large satellites and one pair with smaller satellites. Riley et al. (1958) confirmed these observations. Giorgi and Bozzini (1969b) reported that the karyotype of *A. bicornis* consisted of 2 Sat pairs, 4 SM pairs, and 1 M pair of chromosomes.

The most common karyotype observed in this study consisted of 1 St pair, 4 SM pairs, and 2 M pairs of chromosomes. On the basis of karyotype morphology, *A. bicornis* could not be the B genome donor.

Feldman (1978) reported that the karyotype of *A. searsii* containted 2 pairs of chromosomes with large satellites. Kushnir and Halloran (1981) concluded that the karyotype of *A. searsii* consists of 2 Sat pairs, 3 SM pairs, and 2 M pairs of chromosomes. This conclusion was based on a single idiogram from Feldman (1978).

The most common karyotype of *A. searsii* observed in this study consisted of 2 Sat pairs, 1 St pair, 3 SM pairs, and 1 M pair of chromosomes. On the basis of karyotype morphology, *A. searsii* could be the B genome donor to tetraploid and hexaploid wheat.

Riley et al. (1958) reported that the karyotype of *A. sharonensis* contained one pair of chromosomes with larger satellites and one pair with smaller satellites. Chennaveeraiah (1960) agreed with these observations and Kushnir and Halloran (1981) observed larger satellites than the previous studies. Kushnir and Halloran (1981) reported that the karyotype consisted of 2 Sat pairs, 1 St pair, 3 SM pairs, and 1 M pair of chromosomes. However, this is based on a single cell from *A. sharonensis*.

The most common karyotype of *A. sharonensis* observed in this study consisted of 2 St pairs, 3 SM pairs, and 2 M pairs of chromosomes. On the basis of karyotype morphology, *A. sharonensis* could not be the B genome donor to wheat.

Giorgi and Bozzini (1969b) concluded that the karyotype of *T. urartu* consisted of 2 Sat pairs, 3 SM pairs, and 2 M pairs of chromosomes.

The most common karyotype observed in this study consisted of 2 Sat pairs, 3 SM pairs, and 2 M pairs of chromosomes. However, there is variation in the karyotype morphology. For example, 1 St pair occurs in 50% of the karyotypes, 3 SM occurs in 60%, and 2 M occurs in 50%. Since this results in a total of 6 chromosome pairs, one group has to be increased. In this case, since 3 SM and 2 M occurred more often than 1 St and 3 M, it was concluded that the most common karyotype of _T_. _urartu_ consisted of 2 St pairs, 3 SM pairs, and 2 M pairs. This observation is consistant with that of Giorgi and Bozzini (1969b) since the two chromosome pairs with small satellites had a St arm ratio. Therefore, it is assumed that the St chromosomes observed in this are the same as the Sat chromosomes observed in the previous study.

On the basis of karyotype morphology, _T_. _urartu_ could not be the B genome donor.

The description of the most common karyotype observed for each species studied is given in Table 22. These results are based on a single line of each species and so caution must be used when considering the strength of the conclusions. However, on the basis of chromosome morphology, _A_. _searsii_ is the only species examined that has a karyotype identical to the B genome in _T_. _turgidum_.

_A_. _searsii_ was the most likely candidate as the B genome donor to _T_. _turgidum_ and _T_. _aestivum_ based on the amino acid sequence of purothionin and the morphology of the somatic chromosome complement. Evidence to support this conclusion comes from a variety of other studies. It had been observed that _A_. _searsii_ contained 2 chromosome pairs with satellites that were morphologically similar to chromosomes 1B and 6B in _T_. _turgidum_ and _T_. _aestivum_ (Feldman, 1977). _A_. _searsii_ grows wild close to populations of _T_. _monococcum_ and _T_. _turgidum_ in Israel, Jordan, and Syria and has had the opportunity to cross in these locations (Feldman, 1978). The *in situ* hybridization pattern of highly repeated DNA sequences from the B genome in *T. turgidum* and *T. searsii* were similar (Peacock, 1981). The restriction pattern of the 5S rRNA genes from the B genome in *T. turgidum* was identical to that found in *A. searsii* (Peacock, 1981). The heteroduplex stability of unique or repeated DNA sequences formed between *T. turgidum* and *A. searsii* indicated a high degree of sequence homology (Thompson and Nath, 1986). Therefore, on the basis of the present study and the studies just mentioned, of all the species examined to date, *A. searsii* appears to be the most likely candidates as the B genome donor of wheat.

	Sat	St	SM	M
T. monococcum	0	1	4	2
T. turgidum	2	2	7	3
B genome donor	2	1	3	1
A. speltoides	2	0	3	2
A. longissima	2	1	4	0
A. bicornis	0	1	4	2
A. searsii	2	1	3	1
A. sharonensis	1	1	4	1
T. urartu	0	2	3	2

Table 22. The description of the most common karyotype observed in this study.

Summary and Conclusions

It is difficult to compare the results of previous work to the results of this present study because few of the previous studies included all potential B genome donor species. For example, based on DNA content studies by Reece (1963), it was concluded the *A. speltoides* was the most likely candidate as the B genome donor to common wheat. However, it must be realized that *A. sharonensis* and *A. searsii* were not included in the study. In other studies, more than one species were implicated as the B genome donor to tetraploid and hexaploid wheat. For example, the restriction pattern of the 5S rRNA repeating unit implicated *A. longissima*, *A. sharonensis*, and *A. searsii* and discluded *A. speltoides* as potential B genome donors to the wheats. In addition, one must bear in mind that intraspecific variation may occur. All studies include only 1 or a few lines of each species and may not represent all the variation that exists in nature. Evidence implicating *A. speltoides* as the B genome donor include *in situ* hybridization of highly repeated DNA sequences (Peacock et al., 1981), heteroduplex thermal stability of repeated DNA formed between *T. turgidum* abd *A. speltoides* Thompson and Nath, 1986), and the isoelectric point of the large subunit of rubisco (Chen et al., 1975). Evidence against *A. speltoides* as the B genome donor is the pairing data in hybrids from *A. speltoides* x *T. turgidum* (Sears and Okamoto, 1958; Riley, 1958; Riley et al., 1961), the restriction pattern of the 5S rRNA repeating unit (Peacock et al., 1981), heteroduplex thermal stability of unique sequences from *T. turgidum* and *A. speltoides* (Thompson and Nath, 1986), restriction pattern of chloroplast DNA (Tsunewaki and Ogihara, 1983), and electrophoretic patterns of endosperm proteins (Johnson, 1975). The amino acid sequence of purothionin and the description of the karyotype indicates the *A*. *speltoides* is not the B genome donor to *T*. *turgidum* and *T*. *aestivum*.

A. *longissima* can also be considered a possible candidate for the B genome donor on the basis of the isoelectric point of the large subunit of rubisco (Chen et al., 1975), the restriction pattern of 5S rRNA repeating unit (Peacock et al., 1981), *in situ* hybridization studies of highly repeated DNA (Peacock et al., 1981), and the restriction fragment pattern of chloroplast DNA (Tsunewaki and Ogihara, 1983). Evidence against *A*. *longissima* as the B genome donor is the lacking of pairing in the hybrids from *A*. *longissima* x *A* *speltoides* (Riley et al., 1958), the morphology of the chromosome pairs with satellites (Riley et al., 1958), electrophoretic pattern of endosperm proteins (Johnson, 1975), and geographical distribution (Tsunewaki and Ogihara, 1983). On the basis of amino acid sequence of purothionin and the karyotype morphology, *A*. *longissima* is not the B genome donor to *T*. *turgidum* and *T*. *aestivum*.

A. *bicornis* can be considered a possible candidate as the B genome donor to wheat on the basis of the morphology of an amphiploid hybrid produced from a cross between *A*. *bicornis* and *A*. *turgidum* (Sears, 1956a). All other published data (see above for references) indicates that *A*. *bicornis* is not the B genome donor to the wheats. Although the amino acid sequence of purothionin from *A*. *bicornis* differs from α_1-purothionin by only 1 amino acid, chromosome morphology indicates that *A*. *bicornis* is not the B genome donor to the wheats.

A. searsii can be considered a possible candidate as the B genome donor to wheat on the basis of *in situ* hybridization of repeated DNA sequences (Peacock et al., 1981), the restriction pattern of the 5S rRNA repeating unit (Peacock et al., 1981), geographical distribution (Feldman, 1977), and heteroduplex stability of repeated DNA, and unique sequences formed between *T. turgidum* and *A. searsii* (Thompson and Nath, 1986). Evidence against *A. searsii* as the B genome donor are the electrophoretic profile of the endosperm proteins (Johnson, 1975) and the restriction fragment pattern of the chloroplast DNA (Tsunewaki and Ogihara, 1983). The amino acid sequence of purothionin from *A. searsii* differs from α_1-purothionin by 1 amino acid substitution and the karyotype description is identical to that expected by the B genome basis. Therefore, *A. searsii* is considered to be the most likely candidate as the B genome donor to *T. turgidum* and *T. aestivum* .

A. sharonensis can be considered a possible candidate as the B genome donor to wheat on the basis of the restriction pattern of the 5S rRNA repeating unit (Peacock et al., 1981) and the morphology of the karyotype (Kushnir and Halloran, 1981). All other reports indicate that *A. sharonensis* could not be the B genome donor to the wheats. The karyotype description that was observed in this study, disagreed with the observation of Kushnir and Halloran (1981). Both the karyotype description and the amino acid sequence of purothionin indicate *A. sharonensis* could not be the B genome donor to wheat.

T. urartu can also be considered a possible candidate as the B genome donor to wheat on the basis of the electrophoretic profile of the endosperm protein (Johnson, 1975), anther morphology (Dhaliwal and Johnson, 1976), and pairing affinities to *T. turgidum* chromosomes (Dhalwal and Johnson, 1982). However, all other reports, including the observations in this study, indicate that *T. urartu* is more closely related to the A genome of tetraploid and hexaploid wheats than the B genome from these species.

A summary of the previous data which has excluded the various putative B genome donors is given in Table 23. *A. searsii* can be excluded on the basis of the restriction pattern of ct DNA only. All other studies that have included *A. searsii* in the analysis has supported the proposal that this species is the most likely candidate at the B genome donor to the polyploid wheats.

Species	**Evidence Which Excludes Various Species as the B Genome Donor to the Polyploid Wheats.**
A. speltoides	1) Meiotic pairing data in hybrids from a cross between *T. turgidum* and *A. speltoides* (Sears and Okamoto, 1958; Riley, 1958; Riley et al., 1961). 2) Electrophoretic pattern of alcohol soluble and water soluble proteins (Johnson, 1975). 3) Restriction patternof 5S rRNA repeating unit (Peacock et al. 1981). 4) Restriction pattern of chloroplast DNA (Tsunewaki and Ogihara, 1983). 5) Heterodupled thermal stability data (Thompson and Nath, 1986).
A. longissima	1) Meiotic pairing data in hybrids from a cross between *T. turgidum* and *A. longissima* (Riley et al., 1958). 2) The morphology of chromosome pairs with satellites (Riley et al., 1958). 3) DNA content (Rees and Walters, 1965). 4) Electrophoretic patternof alcohol soluble and water soluble proteins (Johnson, 1975). 5) Heterodupled thermal stability data (Thompson and Nath, 1986).
A. bicornis	1) Meiotic pairing data in hybrids from a cross between *T. turgidum* and *A. bicornis* (Riley et al., 1958). 2) The morphology of chromosome pairs with satellites (Riley et al., 1958). 3) DNA content (Rees and Walters, 1965). 4) Isoelectric point of the large subunit of rubisco (Hirai and Tsunewaki, 1981). 5) Restriction pattern of ct DNA (Tsunewaki and Ogihara, 1983). 6) Heteroduplex stability data (Thompson and Nath, 1986).
A. searsii	1) Restriction pattern of ct DNA (Tsunewaki and Ogihara, 1983).
T. urartu	1) Isoelectric point of the large subunit of rubisco (Chen et al., 1975). 2) Meiotic pairing in a hybrid between *T. urartu* and *T. turgidum* (Dvorak, 1976; Chapman *et al.*, 1976). 3) *In situ* hybridization of repeated DNA sequences from the B genome in *T. turgidum* (Peacock et al, 1981). 4) Heteroduplex stability data (Nath et al., 1983).

(Table 23 continued)

Species	**Evidence Which Excludes Various Species as the B Genome Donor to the Polyploid Wheats.**
A. sharonensis	1) Meiotic pairing in hybrids from a cross between T. turgidum and A. sharonensis (Riley et al., 1958). 2) The morphology of chromosome pairs with satellites (Riley et al., 1958). 3) Electrophoretic patternof alcohol soluble and water soluble proteins (Johnson, 1975). 4) Isoelectric point of the large subunit of rubisco (Hirai and Tsunewaki, 1981). 5) Restriction patternof ct DNA (Tsunewaki and Ogihara, 1983). 6) Heterodupled thermal stability data (Thompson and Nath, 1986).

Table 23. Evidence which excludes the above species as the B genome donor to T. turgidum and T.aestivum.

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